NUTRITION RESEARCH

The International Medium
for Rapid Publication of Communications
in the Nutritional Sciences

List of Contents, Author and
Key Word Index
for Volume 1, 1981

PERGAMON PRESS
New York / Oxford / Toronto
Paris / Frankfurt / Sydney

NUTRITION RESEARCH

(ISSN 0271-5317)

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Pergamon Press, Maxwell House, Fairview Park, Elmsford, New York 10523, USA, and Headington Hill Hall, Oxford OX3 0BW, England.

PUBLISHED BIMONTHLY. *Annual Subscription Rate* for libraries, university departments, government laboratories, industrial and other multiple-reader institutions: (1982)US$75.00. Two-year rate (1982/83)US$142.50. *Special Reduced Rate for Individuals* whose libraries subscribe: US$30.00. *Special rate for members* of the American Institute of Nutrition, the American Society for Clinical Nutrition or any national nutrition society: US$20.00. *Special rate for students:* US$18.00. Prices include surface postage and insurance. Air mail subscriptions extra.

Microform Subscriptions and Back Issues: Current subscriptions are available in microfiche and microfilm. Back issues of all previously published volumes are available in both regular hard copy editions and in microform. Prices supplied upon request from Pergamon Press and/or its division, Microforms International Marketing Corporation, Fairview Park, Elmsford, New York 10523, USA, or Headington Hill Hall, Oxford OX3 0BW, England.

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NUTRITION RESEARCH, Vol. 1, p. 1, 1981
0271-5317/81/010001-01$02.00/0 Printed in the USA.
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EDITORIAL

More Thought on Food

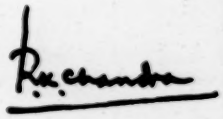
Nutrition impinges on a broad spectrum of biological investigation and clinical practice. There is a growing recognition of the singular importance of food as a determinant of disease, disability and death. The discipline of nutrition includes the study of multiple facets of nutrients in foods from the time they are ingested to the time their metabolic products are either assimilated or excreted. It includes the observation of sociocultural and anthropologic factors influencing food availability and intake. Dietary factors are now established to be important regulators of work performance, morbidity and survival.

The science of nutrition is not new. However, in recent years there has been a tremendous resurgence in interest in the influence of diet on health and concommitantly a logarithmic increase in nutrition research. And yet there is no suitable forum where information generated from such investigations could be rapidly published and the data made available to other research workers, health professionals, nutritionists, dieticians, and planners.

Nutrition Research is being initiated to fill this void. The Journal will rapidly publish original papers and review articles covering basic and applied research on all aspects of nutritional sciences. It will include articles on nutritional biochemistry and metabolism; nutrient requirements in health and disease; digestion and absorption of foods; nutritional anthropology and epidemiology; the influence of socioeconomic, cultural and political factors on nutrition of the individual and the community; the impact of nutrient intake on disease response, work performance and behavior; the consequences of nutritional deficiency on growth and development, endocrines, nervous system and immunity; food intolerance and allergy; nutrient drug interactions; nutrition and ageing; nutrition and cancer; obesity; and intervention programs. Papers on nutritional research in both man and animals will be considered for publication. Each manuscript will undergo prompt and careful peer review. Accepted articles will be included in the next number of the Journal.

Nutrition Research is privileged to have on its Editorial Board an international panel of distinguished scientists. In addition, the Journal will draw upon the expertise and help of other experts to review manuscripts. Besides original articles and critical reviews, invited commentaries, reviews of books, summaries of conferences, announcements, abstracts and letters to the Editor will be published.

The academic success of a scientific journal depends largely upon the quality of material received for publication. Nutrition Research is being launched with the fond hope that the Journal will become the foremost international medium for rapid communication in nutritional research. Comments by contributors and readers will be greatly appreciated and will mould the future shape of this new publication.



Cambridge, Massachusetts
February 2, 1981

NUTRITION RESEARCH, Vol. 1, pp. 3-11, 1981
0271-5317/81/010003-09$02.00/0 Printed in the USA.
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THE ROLE OF INSULIN AND PARATHYROID HORMONE IN THE PROTEIN-INDUCED CALCIURIA OF MAN.[1-3]

Lindsay H. Allen, Ph.D., Geoffrey D. Block, M.S.
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ABSTRACT

The consumption of dietary protein has been shown to increase urinary calcium due to a reduction in the renal tubular reabsorption of calcium. This experiment was designed to examine the post-prandial effects of consuming adequate or high protein meals on serum insulin and parathyroid hormone (PTH), and the relationships between the secretion of these hormones and urinary calcium, phosphorus, sodium, and potassium. Six female and five male adults were each fed meals containing either 15 g protein (control, C) or 45 g protein (high protein, HP). The C and HP diets contained similar amounts of minerals and lactose, and calorie content was equalized by adding fat to the control diet. Serum and urine samples were collected during 4 hours after meal consumption. As reported in a previous publication, urinary calcium was significantly increased between 1.0 and 2.0 hours after consuming the HP compared to the C meal, due to a significant reduction in renal tubular calcium reabsorption. Serum insulin values were significantly increased by the HP compared to the C meal between 0.5 and 1.5 hours. For each subject, we compared the percent increase in calcium excretion and serum insulin after the HP meal compared to the C meal, and found a significant correlation ($r=0.61$, $P<0.05$). Therefore, insulin may be involved in the calciuretic response to protein ingestion. After the consumption of either meal serum PTH fell rapidly and was higher at 1.5 hours after the HP compared to the C meal. Change in serum PTH was not correlated with change in any other parameter including urinary calcium and phosphorus excretion. Despite the post-prandial fall in serum PTH, urinary phosphorus increased steadily throughout the 4 hours. Dietary protein had no effect on urinary phosphorus or renal tubular phosphorus reabsorption. Urinary sodium and potassium excretion were lower after the HP meal, possibly because of the increased insulin secretion. Females tended to show greater post-prandial changes in urinary calcium and serum insulin.
KEY WORDS: Protein, calcium, insulin, parathyroid hormone, phosphorus

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1 Supported by DE-0429 from the United States Public Health Service
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INTRODUCTION

It has been 40 years since it was first shown that increasing the protein content of the diet causes an increase in urinary calcium excretion (1). During the last decade, several investigators have attempted to identify the mechanism of protein-induced calciuria, using both animal models (2,3) and human subjects (4-9). It has been established that the calciuria is not caused by an increase in the intestinal absorption of calcium (6), and that it is independent of the level of dietary calcium over a wide range of protein intakes (5). More recently we have shown that the calciuria is caused by a decrease in the renal tubular reabsorption of calcium which occurs rapidly after the consumption of a single meal containing protein (8).

Our most recent experiments have been designed to determine the reason why renal reabsorption of calcium is reduced. We have shown that the sulfur amino acid content of the protein is not responsible for the impairment in calcium reabsorption, since urinary calcium excretion is not affected by the sulfur amino acid content of the diet, and is not related to urinary sulfate, net acid or titratable acid excretion (10).

Both insulin (11,12) and PTH (13) have been shown to affect urinary calcium excretion. In previous experiments on protein-induced calciuria, fasting serum levels of insulin (6) and PTH (6,9) were found to be unaffected by level of dietary protein. However, we have shown that the maximum calciuretic effect of dietary protein occurs between 0.5 and 3.0 hours after ingestion of a protein-containing meal (8). In the experiment reported here, we have measured changes in serum insulin and PTH during this time period in order to determine whether inter-subject differences in the degree of calciuretic response to protein are related to differences in insulin or PTH secretion. Postprandial changes in phosphorus, sodium, and potassium are also reported, since insulin has been shown to decrease the urinary excretion of phosphorus, sodium, and potassium (11,12) while PTH increases the urinary excretion of phosphorus (14).

METHODS

Our subjects were six female and five male healthy adults. Liquid formula diets provided either 15 g protein (control, C) or 45 g protein (high protein, HP) per meal. The composition of the diets is shown in Table 1. Each meal contained 25% of the Recommended Dietary Allowance for calcium, phosphorus, and magnesium. The amount of sodium, potassium, sucrose and lactose was adjusted so that it was equal in both diets. Additional fat was added to the control diet to equalize calorie content. After the diets were prepared, aliquots were wet-ashed (15) and analyzed for calcium and zinc by atomic absorption, nitrogen (16), and sodium and potassium by flame photometry. Measured values were within ±5% of calculated values.

Each subject consumed the two meals on separate occasions, one week apart. The order of receiving the meals was randomly assigned. On the day preceding the test meal, the subjects consumed foods from a standardized menu of normal foods, and fasted from 2130 hours. On waking, each subject voided completely, recorded the time and drank 300 ml water. Fasting blood and urine samples were obtained at 0845 hours, and the meal was consumed at 0900 hours. After meal consumption, and at every 0.5 hours for the next 4 hours, each subject drank 200 ml water so that a steady, moderate diuresis was maintained. Additional blood samples were taken at 0.5, 1.0, 1.5, 2.5, and 3.5 hours, and urine samples every 0.5 hour for 4.0 hours after each meal.

TABLE 1
Composition of Diets

Ingredient	Control	High protein
	- - - g/meal - - -	
Protein[a]	15.0	45.0
Sucrose[b]	20.0	20.0
Lactose[c]	0.172	0
Safflower oil[d]	21.0	7.0
$CaCl_2 \cdot H_2O$	0.549	0
H_3PO_4[c]	1.066	0
NaCl[e]	0.628	0.614
KCl[c]	1.052	0
MgO[e]	0.116	0.101
Zn acetate	0.009	0
H_2O	350.0	350.0

[a] [b] Crest Foods Co., Inc., Ashton, IL Amstar Corp; American Sugar Div., New York, N.H., [c] Fisher Scientific Co., Fairlawn, N.J., [d] Hollywood Health Foods, Los Angeles, CA, [e] J.T. Baker Chemical Co., Phillipsburg, N.J. [f] Eastman Kodak Co., Rochester, N.Y.

Urine samples were analyzed for calcium (17), phosphorus (18), and sodium and potassium by flame photometry. Serum analyses included insulin[4] and parathyroid hormone[5] by radioimmunoassay. Data on urinary pH, titratable acid, ammonium, calcium, and sulfate, serum ionic and total calcium and glomerular filtration rate have been reported in a previous publication (10).

Analysis for sex and diet effects was achieved by analysis of variance on a repeated measures model (19). For all parameters measured, the paired t-test was used to determine the effects of consuming HP *vs* C (20). Each subject acted as his or her own control. Regression analysis (21) was used to measure the linear relationships between variables. Significance levels are shown at the top of each figure, and data are plotted as the mean ± standard deviation.

RESULTS

As previously reported (10) the level of dietary protein had no significant effect on urine flow rate, pH, glomerular filtration rate, total or filterable serum calcium, or urinary ammonium, net acid or titratable acid. However, urinary calcium excretion was significantly higher between 1.0 and 2.0 hours after consuming the HP compared to the control meal, due to a reduction in the fractional renal tubular reabsorption of calcium.

Serum insulin levels (Figure 1) peaked at 0.5 hour after meal consumption, and were significantly increased by the HP compared to the control meal from 0.5 to 1.5 hours. For each subject, we calculated the percent increase in serum insulin concentration and in urinary calcium excretion after the HP meal with respect to the C meal (Figure 2). Linear regression analysis revealed a

[4] Phadebas Insulin Test, Pharmacia Diagnostics, Piscataway, N.J.
[5] Analyses performed by G. Bryce at Hoffman-LaRoche Inc., using human serum standard and CH/9 antiserum. One µl equivalent = 10 pg bovine PTH.

significant correlation (r=0.61, P<.05).

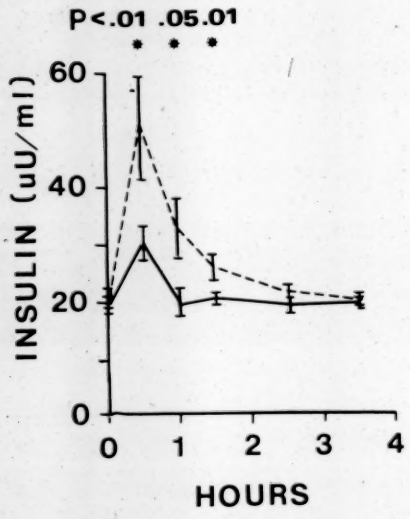

FIG.1
Serum insulin concentration after C (-) and HP (--) meals.

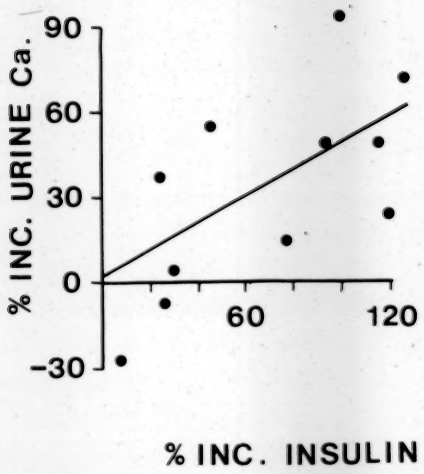

FIG. 2
Regression of percent increase in urine calcium against percent increase in serum insulin.
Y=0.48X-3.0, r = 0.61, P<.05

Serum parathyroid hormone (PTH) values are shown in Figure 3. There was a marked reduction in PTH levels following the consumption of both diets. Compared to the control meal, values were significantly greater at 1.5 hour after the HP meal. No significant correlations were found between change in serum PTH and change in any other parameter, including urinary calcium and phosphorus excretion, after either meal. Urinary phosphorus excretion increased steadily throughout the 4 hours after meal consumption, but was not significantly affected by the level of dietary protein (Figure 4). Serum phosphorus also increased throughout the experiment, and was significantly lower at 1.0 and 1.5 hours after the HP meal compared to the control. However, dietary protein level had no effect on the amount of phosphorus filtered or reabsorbed.

At 1.0 and 1.5 hours after the HP meal, the urinary excretion of both sodium and potassium was lower than after the control meal, but because of the large individual variation the only significant reduction was in urinary potassium at 1.0 hour (Figures 5 and 6).

Analyses of variance indicated significant difference due to sex and/or sex X time interaction for several variables. Females had lower fasting urinary phosphate and serum PTH. After meals, females had a greater and more rapid increase in urinary calcium than males, although total urinary calcium for the 4 hour period was similar. The female subjects, as a group, had a higher mean increase in postprandial insulin levels than males, but this difference was not significant. Females also had a significantly lower fractional calcium reabsorption across time and a greater fractional reabsorption of phosphorus. At 1.5 hours after the HP meal, the fractional reabsorption of phosphate was

increased by more than 100% relative to males.

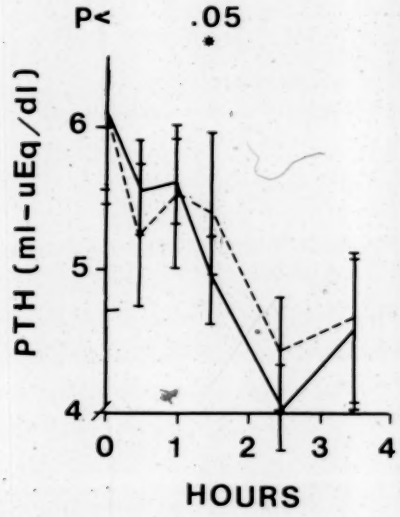

FIG. 3
Serum PTH concentration after C (-) and HP (--) meals.

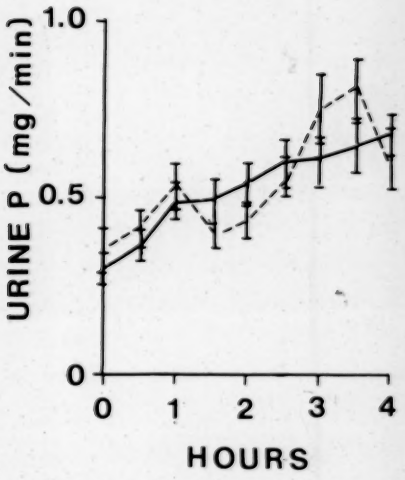

FIG. 4
Urinary phosphorus excretion after C (-) and HP (--) meals.

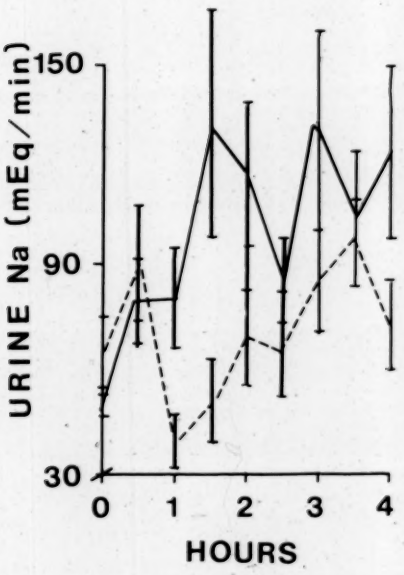

FIG. 5
Urinary sodium excretion after C (-) and HP (--) meals.

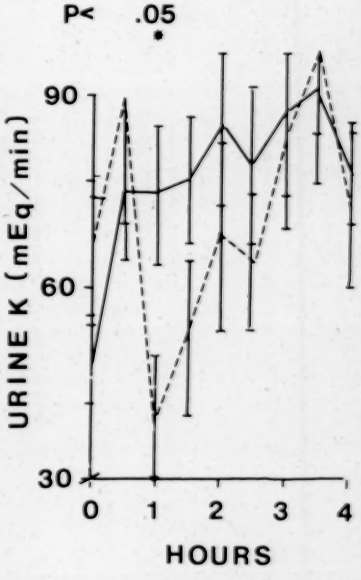

FIG. 6
Urinary potassium excretion after C (-) and HP (--) meals.

DISCUSSION

Consumption of either the control or high protein meal caused rapid changes in the metabolism of calcium, phosphorus, insulin, and PTH. The excretion of both calcium and phosphate in urine was rapidly increased, due to a decrease in their fractional renal reabsorption. Consumption of the HP diet compared to C caused an even greater reduction in calcium reabsorption, but had no significant effect on phosphate reabsorption. Serum PTH levels fell rapidly after both meals. PTH has been shown to simultaneously decrease phosphate reabsorption in the proximal tubule, and stimulate calcium reabsorption in the distal tubule (22,23). However, in this experiment, rapidly falling serum PTH levels accompanied a decrease in the reabsorption of both calcium and phosphate. The pronounced calciuria induced by the high protein diet did not occur as a result of reduced PTH secretion; serum levels of PTH were actually significantly higher at 1.5 hours after the HP meal.

It is well established that serum insulin levels increase in response to either carbohydrate or protein ingestion (24,25). If protein is ingested together with carbohydrate, there is a synergistic effect on insulin production which results in an exaggerated rise in serum insulin (25). Since the meals were made isocaloric by adjusting fat content in the present experiment, the total amount of protein plus carbohydrate was lower in the control compared to the high protein diet. This fact may have explained some of the greater increases in serum insulin after the HP meal. Of great interest was the fact that for each subject, the increment in peak serum insulin levels, when HP was compared to C, was strongly correlated with the increase in urinary calcium. In a previous experiment, we found that some subjects showed a negligible calciuric response to a high protein intake (8). In the present experiment, we observed the same phenomenon. Two of our 11 subjects showed no increase in urinary calcium after consuming HP compared to C; these 2 subjects were also those who showed the smallest increase in serum insulin.

From the data obtained from this experiment, we have obtained the observation that urinary calcium excretion is correlated with serum insulin levels. While we have no explanation for this relationship at present, we are investigating the possibility that insulin might interfere with the action of PTH on calcium reabsorption. Using rat renal cortex slices, Northrup (26) recently demonstrated that insulin prevented the PTH stimulation of cAMP and protein kinase production.

DeFronzo has established in humans that insulin infusion, while keeping plasma glucose constant, causes an impairment of calcium reabsorption, and an increase in phosphorus, sodium, and potassium reabsorption (11,12). The greater increase in serum insulin after the HP meal may therefore explain the decreases in urinary sodium and potassium, which occurred 30 minutes after the insulin peak. Schuette et al. also observed that a high protein diet causes a decrease of urinary sodium excretion, but does not affect urinary phosphorus (9). The reciprocal relationship between urinary sodium and calcium excretion observed here is consistent with an effect of insulin on the distal nephron, since the transport of these minerals in the proximal tubules is usually closely associated (11).

If serum insulin affects urinary calcium excretion, this may also explain the observation that the ingestion of metabolizable carbohydrate, alcohol or protein results in an increase in urinary calcium (27,28). In contrast, a calciuric response was not observed after an oral glucose load was given to diabetics (29).

The sex-related differences seen in this experiment should be confirmed in more subjects. Females have a greater increase in serum insulin as a result of consuming protein alone (30). Our female subjects showed significantly lower fasting serum insulin and urinary calcium levels. While there was a tendency to higher postprandial responses of insulin and urinary calcium in females, these differences were not significantly different from those in males. Inter-subject differences in calciuric response are potentially significant in the etiology of the bone loss which occurs as a result of aging.

REFERENCES

1. McCANCE, R.A., WIDDOWSON, E.M. and LEHMANN, H. The effect of protein intake on the absorption of calcium and magnesium. Biochem. J., 36:689-691, 1942.

2. BELL, R.R., ENGLEMAN, D.T., SIE, T.-L. and DRAPER, H.H. Effect of a high protein intake on calcium metabolism in the rat. J. Nutr., 105:475-483, 1975.

3. ALLEN, L.H. and HALL, T.E. Calcium metabolism, intestinal calcium-binding protein and bone growth of rats fed high protein diets. J. Nutr., 103:929-936, 1973.

4. JOHNSON, N.E., ALCANTARA, E.N. and LINKSWILER, H. Effect of level of protein intake on urinary and fecal calcium and calcium retention of young adult males. J. Nutr., 100:425-430, 1970.

5. MARGEN, S., CHU, J-Y., KAUFMANN, N.A. and CALLOWAY, D.H. Studies in calcium metabolism. I. The calciuretic effect of dietary protein. Amer. J. Clin. Nutr., 27:584-589, 1974.

6. ALLEN, L.H., ODDOYE, E.A. and MARGEN, S. Protein-induced hypercalciuria: a longer term study. Amer. J. Clin. Nutr., 32:741-749, 1979.

7. SPENCER, H., KRAMER, L., OSIS, D. and NORRIS, C. Effect of a high protein (meat) intake on calcium metabolism in man. Amer. J. Clin. Nutr. 31:2167-2180, 1978.

8. ALLEN, L.H., BARTLETT, R.S. and BLOCK, G.D. Reduction of renal calcium reabsorption in man by consumption of dietary protein. J. Nutr., 109:1345-1350, 1979.

9. SCHUETTE, S.A., ZEMEL, M.B. and LINKSWILER, H.M. Studies on the mechanism of protein-induced hypercalciuria in older men and women. J. Nutr., 110: 305-315, 1980.

10. BLOCK, G.D., WOOD, R.J. and ALLEN, L.H. A comparison of the effects of feeding sulfur amino acids and protein on urine calcium in man. Amer. J. Clin. Nutr., 33:2128-2136, 1980.

11. DeFRONZO, R.A., COOKE, C.R., ANDRES, R., FALOONA, G.R. and DAVIS, P.J. The effect of insulin on the renal handling of sodium, potassium, calcium and phosphate in man. J. Clin. Invest., 55:845-855, 1975.

12. DeFRONZO, R.A., GOLDBERG, M. and AGUS, Z.S. The effects of glucose and insulin on renal electrolyte transport. J. Clin. Invest., 58:83-90, 1976.

13. PEACOCK, M., ROBERTSON, W.G. and NORDIN, B.E.C. Relation between serum and urinary calcium with particular reference to parathyroid activity. Lancet

1:384-386, 1969.

14. ANAST, C.S., CARNES, D.L., NICKOLS, G.A. and FORTE, L.R. Interrelationship of parathyroid hormone and vitamin D. In: Pediatric Diseases Related to Calcium. H.F. DeLuca and C.S. Anast (eds). Elsevier, New York, 1980, pp. 75-105.

15. REITZ, L.L., SMITH, W.H. and PLUMLEE, M.P. A simple, wet oxidation procedure of biological materials. Anal. Chem., 32:1728, 1960.

16. TRIEBOLD, H.D. Quantitative Analysis with Applications to Agricultural and Food Products. D. Van Ostrand Co., New York. 1946, p. 151.

17. Technicon Autoanalyzer II, Clinical Method No. 3A, (1972). Technicon Instruments Corporation, Tarrytown, New York.

18. FISKE, C.H. and SUBBAROW, Y. The colorimetric determination of phosphorus. J. Biol. Chem., 66:375, 1925.

19. Biomedical Computer Programs P-Series. W.J. Dixon and M.B. Brown (eds). University of California Press, Berkeley, 1979, p. 556.

20. Biomedical Computer Programs P-Series. W.J. Dixon and M.B. Brown (eds). University of California Press, Berkeley, 1979, p. 182.

21. SNEDECOR, G.W. and COCHRAN, W.G. Statistical Methods. Iowa State University Press, Ames, Iowa, 1967, p. 135.

22. AGUS, Z.S., GARDNER, L.B., BECK, L.H. and GOLDBERG, M. Effects of parathyroid hormone on renal tubular reabsorption of calcium, sodium, and phosphate. Am. J. Physiol. 224:1143-1148, 1973.

23. GREGER, R., LANG, F. and OBERLEITHNER, H. Distal site of calcium reabsorption in the rat nephron. Pflunger Arch., 374:153-157, 1978.

24. FUJITA, Y., GOTTO, A.M. and UNGER, R.H. Basal and postprotein insulin and glucagon levels during a high and low carbohydrate intake and their relationships to plasma triglycerides. Diabetes, 24:552-558, 1975.

25. RABINOWITZ, D., MERIMEE, T.J., MAFFEZZDI, R. and BURGESS, J.A. Patterns of hormonal release after glucose, protein and glucose plus protein. Lancet, ii:454-457, 1966.

26. NORTHRUP, T.E., KREZOWSKI, P.A., PALUMBO, P.J., KIM, J.K., HUI, Y.S.F. and DOUSA, T.P. Insulin inhibition of hormone-stimulated protein kinase systems of rat renal cortex. Am. J. Physiol., 236:E649-654, 1979.

27. LINDEMAN, R.D., ADLER, S., YIENGST, M.J. and BEARD, E.S. Influence of various nutrients on urinary divalent cation excretion. J. Lab & Clin. Med., 70:236-345, 1967.

28. KALBFLEISCH, J.M., LINDEMAN, R.D., GINN, H.E. and SMITH, W.O. Effects of ethanol administration on urinary excretion of magnesium and other electrolytes in alcoholic and normal subjects. J. Clin. Invest. 42:1471-1475, 1963.

29. MONNIER, L., COLLETTE, C., AGUIRRE, L., -SANY, C. and MIROUZE, J. Intestinal and renal handling of calcium in human diabetes mellitus: influence of acute oral glucose loading and diabetic control. Eur. J. Clin. Invest.

8:225-231, 1978.

30. MERIMEE, T.J., BURGESS, J.A. and RABINOWITZ, D. Sex-determined variation in serum insulin and growth hormone response to amino acid stimulation. J. Clin. Endocrin., 26:791-793, 1966.

NUTRITION RESEARCH, Vol. 1, pp. 13-24, 1981
0271-5317/81/010013-12$02.00/0 Printed in the USA.
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ZINC NUTRITURE AND TASTE ACUITY IN PATIENTS WITH CYSTIC FIBROSIS[1,2]

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ABSTRACT

An assessment of zinc nutriture was undertaken in 19 juvenile patients with cystic fibrosis and 40 control adolescents. Plasma zinc concentration, hair zinc content and quantitative taste acuity score were determined in each individual. In the patients, these data were related to other clinical data. No difference in plasma zinc between patients and controls was found. Moreover, no correlation between biochemical indices of zinc status and growth attainment was seen in contradistinction to a previously reported suggestion that growth-retarded patients were more prone to zinc depletion. Hair zinc was significantly lower and taste acuity thresholds significantly higher in the patients with cystic fibrosis. The impairment in taste acuity contrasts with an earlier report on taste in patients with this disease. Hair zinc levels declined as forced vital capacity of the lungs decreased as a consequence of the pulmonary disease. From the evidence presented in this investigation and in previously reported studies, zinc metabolism appears to be altered in cystic fibrosis; it is likely that impaired zinc nutriture occurs more commonly in patients afflicted with cystic fibrosis than in the population at-large.

KEY WORDS: Cystic fibrosis, Zinc, Taste acuity, Growth retardation, Trace minerals, Hair analysis.

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[1]Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the United States Department of Agriculture, and does not imply its approval to the exclusion of other products that may be suitable.

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INTRODUCTION

Cystic fibrosis is associated with growth retardation, delayed sexual maturation, and short stature (1). The nutritional deficiency of zinc has been assoicated with a syndrome of dwarfism and hypogonadism among rural populations of children in the Middle East (2,3) and with such clinical entitities as sickle cell disease (4) and Crohn's disease (5,6) which are also associated with retardation of growth and development in some affected children. Severe zinc depletion was found in a patient with cystic fibrosis and renal disease reported by Dodge and Yassa (7). Evidence for zinc deficiency among a population of patients with cystic fibrosis was found by Halsted and Smith (8) but not by Palin and associates (9). Interestingly, decreased plasma zinc concentration in the report by Halsted and Smith (8) was only significant in patients with "moderate to severe growth retardation" but not in patients of normal stature and growth.

Hypogeusia or impaired taste acuity has been suggested to be a pathophysiological consequence of zinc deficiency (10,11). Zinc supplementation has resulted in improved taste acuity in several studies (11-15). Taste acuity has also been investigated in patients with cystic fibrosis. Earliest publications reported *increased* taste acuity in patients with cystic fibrosis (16), but subsequent studies have reported normal or subnormal taste thresholds (9,17-20). The explicit relationship between zinc nutriture and taste acuity in cystic fibrosis has only once been investigated (9). We report here our observations on the zinc nutriture of 19 juvenile patients with cystic fibrosis with special attention to growth attainment and taste acuity.

PATIENTS

Nineteen patients, ranging in age from 6 to 17 years, were selected from among registrants at the Cystic Fibrosis Clinic of the Wyler Children's Hospital, University of Chicago Hospitals and Clinics, Chicago, Illinois. Cystic fibrosis had been diagnosed in all patients on the basis of abnormal electrolyte concentrations in sweat and a typical clinical evolution. Patients were studied during routine clinic visits. The biochemical indices of nutriture, exclusive of zinc, in these subjects is the substance of a companion report (21) and the features of vitamin A transport in these patients has been reported elsewhere as well (22).

All patients had some degree of chronic pulmonary disease but were free of clinically apparent acute infections on the day of study. None had a history of any neurological disorder that could have accounted for cranial nerve abnormalities. All but two patients were receiving replacement therapy with pancreatic enzymes to compensate for pancreatic insufficiency. We obtained informed consent from the patients and their parents after explaining the nature, purpose and risks of the study in full. Forty adolescent controls from a private day-school were studied after prior written consent had been obtained from the students' parents.

METHODS

Post-prandial blood samples were drawn between 10 a.m. and noon from

all subjects. Samples were drawn into plastic syringes, placed in plastic, trace metal-free containers containing zinc-free oxalate anticoagulant, and centrifuged promptly. Hemolyzed samples were not used for zinc analysis. Zinc was measured by flame atomic absorption spectrophotometry after the method of Sinha and Gabrieli (23). Hair was taken from the occipital scalp with stainless steel scissors. The proximal 2-3 cm were analyzed after being cut into pieces less than 1 mm in length and washed with acetone, ether, and detergent after the method of Klevay (24). Samples of 15-20 mg were then wet-ashed with nitric and sulfuric acids. The digestate was diluted to 5 ml, and zinc was measured via flame atomic absorption spectrophotometry with 5 volume percent H_2SO_4 matrix for both samples and calibration standards. Digestates of the two brands of pancreatic enzymes (see below) were also analyzed by atomic absorption spectrophotometry to determine their zinc content.

Taste acuity was assessed by the modification of the three-drop, forced-choice dilution technique published by Hambidge et al. (11). In this procedure, tastant solutions representing salty (sodium chloride), sweet (sucrose), bitter (urea) and sour (hydrochloric acid) taste qualities were employed. The concentration matrix is illustrated in Table I. For each dilution, 3 drops were presented to alternate sides of the tongue in a changing order; two drops were distilled, deionized water, and one was the tastant solution. The subject was asked to identify which drop was different from the water; this constituted taste *detection*. The subject was also asked to identify its taste quality; this constituted taste *recognition*. From these responses, detection and recognition thresholds for the individual tastants were calculated. For the purpose of statistical manipulation and comparison, the taste threshold patterns were given a quantitative numerical score by assigning a value of *minus* one for each of the tastant dilutions which could not be correctly distinguished from water (detection score), or by assigning a *minus* one for each error in detection and each error in recognition (combined detection/recognition score).

TABLE I

Concentrations of Tastant Solutions in mmoles

NaCl	30	60	90	150	300
Sucrose	15	30	60	150	300
Urea	120	150	300	500	1000
HCl	3	6	15	30	60

Pulmonary function tests including forced vital capacity (FVC) and the first-second forced expiratory volume (FEV_1) were performed with a Pulmonar^R spirometer (Jones Medical Instrument Company, Oakbrook, Illinois).

Height-for-age percentiles and weight-for-age percentiles for each patient were calculated from the anthropometric scales of the Iowa Child Welfare Research Station and the Stuart curves of the Harvard School of Public Health.

The Student "t" test was used for statistical comparison of mean values between groups of patients or between patients and controls. Simple linear regression analysis was used for calculation of correlation coefficients in the analysis of intragroup data.

RESULTS

The values for age, sex, height-for-age, weight-for-age, pulmonary function tests, and the number of capsules of pancreatic enzymes[6] taken per day are given in Table II. Ten of 19 patients had heights at or below the 10th percentile with 3 at or below the 3rd percentile; in 13, weights were at or below the 10th percentile with 11 at or below the 3rd percentile. Thus, consistent with earlier observations (1) cystic fibrosis patients show a greater relative retardation in weight than in height.

Plasma and Hair Zinc: The mean plasma zinc concentration for the cystic fibrosis group was 71.7 ± 12 µg/dl (mean ± 1 SD) and did not differ significantly from the 78.6 ± 17.7 µg/dl of the adolescent controls. Differences in hair zinc content, however, were highly significant (Figure 1). The mean hair zinc concentration for the cystic fibrosis group was 117 ± 36 µg/g and for the control group was 162 ± 23 µg/g ($p<0.001$). In addition, 8 or 18 patients had levels of hair zinc which were below the entire concentration range of the control group.

TABLE II

SOME CLINICAL CHARACTERISTICS OF THE CYSTIC FIBROSIS PATIENTS STUDIED

Patients		Growth Attaintment		Pulmonary Function		Treatment
age	sex	height percentile	weight percentile	F.V.C.† % of predicted	F.E.V.1* F.V.C.	enzyme caps per day
6y 1m	M	90th	75th	81	78	7
7y 0m	M	80th	90th	82	71	5
8y 3m	F	10th	5th	N.A.¶	N.A.	10
9y 0m	M	35th	30th	79	93	12
9y 5m	M	<3rd	<3rd	71	75	10
9y 5m	M	50th	25th	88	75	12
9y 9m	M	25th	3rd	100	55	3
11y 2m	F	10th	<3rd	92	76	10
11y 3m	F	5th	<3rd	70	66	20
11y 10m	M	15th	<3rd	48	67	26
12y 0m	M	3rd	<3rd	78	43	9
12y 0m	F	50th	<3rd	41	54	16
12y 7m	M	10th	10th	63	71	28
12y 8m	F	10th	20th	98	77	0
13y 6m	M	15th	10th	80	78	15
14y 3m	M	<3rd	<3rd	73	57	12
14y 11m	F	3rd	<3rd	61	53	3
16y 9m	F	15th	3rd	54	45	10
17y 11m	M	10th	3rd	95	88	0

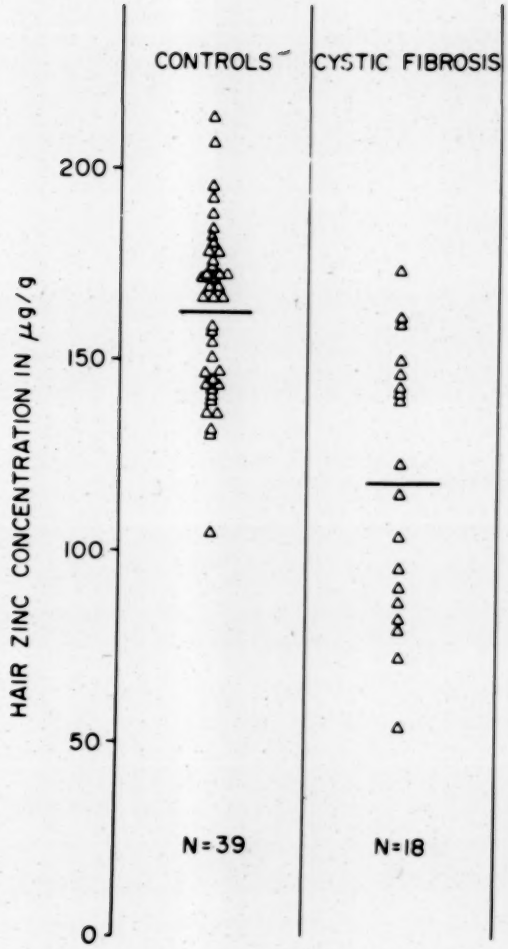
† F.V.C. = forced vital capacity

* $F.E.V._1$ = 1-second forced expiratory volume

¶ N.A. = not available

[6] Viokase® (Viobin Corporation, Monticello, Illinois), or Cotazyme® (Organon, Inc., West Orange, New Jersey).

FIG 1



Hair zinc concentrations in healthy adolescent controls and in cystic fibrosis patients.

The mean plasma and hair zinc concentrations for the cystic fibrosis group, arranged by growth percentiles, are shown in Table III. The standards to classify "moderate to severe growth retardation" used to stratify their population of patients were not reported by Halsted and Smith (8). We arbitrarily used height at or below the 10th percentile and weight at or below the 3rd percenitle as our criterion for biologically significant deficits in linear and ponderal growth, respectively. As seen in Table III, none of the differences between these rather small, but evenly divided, sub-

groups was significant although there is a *suggestion* of a trend toward lower mean plasma zinc concentration in the underweight patients. Linear regression of height of weight percentiles with hair or plasma zinc concentration failed to show statistically significant correlation coefficients.

TABLE III

Mean ± One Standard Deviation of Zinc Data According to the Patients' Weight and Height Percentiles

		plasma zinc concentration (µg/dl)	hair zinc concentration (µg/g)
weight percentile	≤ 3rd	68.4 ± 11.4	122.5 ± 37.2
	≥ 3rd	77.0 ± 11.0	110.8 ± 34.7
height percentile	≤ 10th	72.4 ± 13.1	126.8 ± 30.0
	≥ 10th	72.0 ± 11.1	107.8 ± 39.8

Taste Acuity: Using numerical scoring, we found that taste acuity thresholds were significantly lower for controls than for patients (Figure 2). The mean taste detection score was 1.7 ± 1.3 for patients and 0.8 ± 1.0 for the controls ($p<0.02$); the combined taste detection and recognition scores were 5.4 ± 2.8 and 2.9 ± 2.4 for the patients and controls, respectively ($p<0.001$). The majority of the errors among the patients were with the sour and bitter tastants. Only 2 of 18 patients tested failed to detect the lowest concentration of NaCl and only 5 of 18 failed to detect the lowest concentration of sucrose.

Clinical Correlates: To determine whether or not any significant correlation between zinc status and clinical impairment attributable to the underlying cystic fibrosis could be discerned, linear regression of hair zinc, plasma zinc and taste score with height, weight, pancreatic enzyme capsule requirement, FEV_1, FVC and the FEV_1/FVC ratio were performed. The only statistically significant correlation was between hair zinc and FVC, the index of restrictive pulmonary disease ($r = 0.534$; $p<0.05$).

Zinc Content of Pancreatic Enzyme Preparations: The zinc concentration in Viokase^R capsules was 145 ± 2 µg/g (mean ± SD: N = 3) giving each capsule a zinc content of 51 µg. The zinc concentration in Cotazyme^R capsules was 112 ± 1 µg/g (N = 4) giving each capsule a zinc content of 55 µg. The greatest consumption of pancreatic enzymes was 28 Viokase^R capsules per day. This would have contributed 1.4 mg of zinc in addition to the dietary intake. This is equivalent to one-seventh to one-eleventh of the child or adolescent daily allowance for this nutrient.

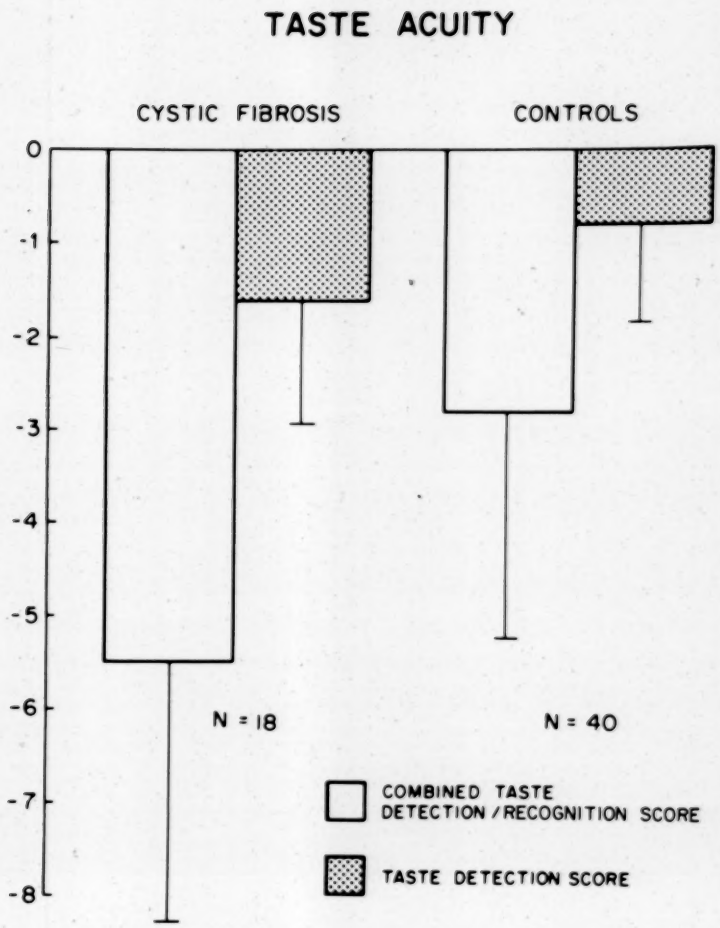


FIG 2

Combined taste detection and taste recognition scores and simple taste detection scores for cystic fibrosis patients and healthy adolescent controls. Mean + 1 SD is represented.

DISCUSSION

Our findings add to the converging evidence that zinc metabolism is disordered in cystic fibrosis (8,9,22,25-27). A problem in assigning a precise description to zinc status in this disease is the lack of reliable assessment tools for defining zinc nutriture. In the past, plasma and serum,

erythrocytes, hair, sweat, saliva, skin, and urine have been used for static tissue or body-fluid determinations of zinc concentration; no one of these indices can be considered unequivocal in reflecting total-body zinc status (28). We included two determinations plasma zinc and hair zinc concentration, in our nutritional evaluation of our patients and controls. Physiological disorders in human zinc depletion include depressed activity of zinc metalloenzymes, impaired wound healing, decreased immune responsiveness, nightblindness, and hypogeusia (28). From these, we chose taste acuity as a potential *functional* index of zinc nutriture.

As in the report by Palin et al. (9), we found no significant difference between the plasma zinc concentration of patients and controls. However, in contrast to the findings of Palin et al. (9) who also found no differential in taste acuity or hair zinc, we found a significantly lower taste acuity score in our patient group, and 10 of 18 patients had hair zinc concentration below the normal range. Palin et al, (9) went on to supplement their patients with 50 mg of zinc sulfate or a zinc-free placebo for 8 weeks. No influence on growth or in clinical indices of zinc nutriture was detected. This latter finding is not unexpected in the absence of strong indications that zinc deficiency was initially present among their clinic population. Our data provide evidence that in *some* juvenile outpatients with cystic fibrosis, zinc nutriture is indeed impaired.

Halsted and Smith (8) found diminished levels of plasma zinc in growth-retarded cystic fibrosis patients. Palin et al. (9) analyzed their plasma zinc data by stature and failed to find an association. We have also been unable to confirm any significant reduction in plasma zinc among growth-retarded patients although a *trend* toward lower zinc concentration with more weight deficit was suggested. It is curious that Halsted and Smith (8) reported that the same group of growth-retarded patients with reduced plasma zinc levels also had more severe pulmonary disease. In bacterial and other infections, leukocytic endogenous mediators (LEM), derived from stimulated neutrophils and macrophages, promote a rapid redistribution of zinc from the circulation into the liver (29,30); we have shown that LEM activity is partially responsible for the lowered zinc concentration in the plasma of hospitalized patients with inflammatory bowel disease (31). The differences observed by Halsted and Smith (8) in plasma zinc concentration between normal stature and growth-retarded patients might have been caused by active or smoldering lower respiratory infections in the latter group. None of our patients was clinically infected at the time of study. Thus, the data available can neither support nor disprove the hypothesis that zinc deficiency is a major cause of the growth retardation observed in cystic fibrosis. The present findings suggest that zinc deficiency is one of several possible contributing factors that might act individually or in concert with other nutritional and non-nutritional factors to retard linear growth or weight gain in this disease.

The significant correlation between restrictive lung disease and low hair zinc might reflect the resultant tissue depletion from previous recurrent episodes of pulmonary infection. Thus, as pulmonary disease advances zinc nutriture may become progressively impaired. It is of note that a significant correlation between FVC and *increasing* copper concentration was found in these patients (21). We found no clinical correlations with the pancreatic enzyme requirement, and we confirm the observation by Palin et al. (9) of an insignificant contribution to total dietary intake of zinc from the zinc content of pancreatic extract capsules.

The effect of cystic fibrosis, *per se*, on taste acutiy is unresolved. Conflicting observations have been reported in patients with this disease. Henkin and Powell (16) studied all of the four basic qualities of taste - salt, sweet, bitter, and sour - in cystic fibrosis patients and age-matched controls; using a variant of the forced-choice dilution technique in which volumes of tastant solutions were sipped and tasted, they found *hyper*geusia

or markedly reduced taste threshold (increased acuity). Concentrations as low as 10^{-5} M were used. Hertz et al. (19) employing identical procedures to those of Henkin and Powell (16) were unable to confirm the observation of hyperquesia; the sensitivity of children with cystic fibrosis fell within the normal range. This was also the general experience of others (9,17,18, 20). In our design, we used a three-*drop* test,and did not present concentrations of NaCl below 3×10^{-2} M. Thus our methods are not strictly comparable to those applied by Henkin and Powell (16) and Hertz et al. (19). Moreover, the majority of decreased taste detection and taste recognition among our patients was experienced with the bitter (urea) and sour (HCl) tastant solutions, reflected in the significant reduction in the *composite* scores for this group. Hertz et al. (19) have noted that the higher basal concentrations of sodium in the saliva of patiens with cystic fibrosis might perturb taste perception, and suggest that as a caveat in taste evaluation in this disease. Furthermore, vitamin A deficiency is known to affect taste acuity. The fact that 8 of our patients had plasma vitamin A concentrations below 30 µg/dl (21,22) might have independently influenced taste acuity. Palin et al. (9) found no improvement in taste acuity with zinc supplementation of their patients, but none exhibited initial hypogeusia. A deeper insight into the relative zinc-dependence of the reduced taste acuity of our patients might have been achieved through zinc supplementation of our population. This was, unfortunately, beyond the scope of our experimental design.

The pathogenesis of impaired zinc metabolism and zinc deficiency in patients with cystic fibrosis is unknown and remains a matter for speculation. Based on specific clinical features of cystic fibrosis, however, we can postulate several mechanisms that might contribute to derangement of zinc metabolism. The first is the possibility of excessive urinary zinc loss from recurrent pulmonary infection in addition to its redistribution under the influence of LEM discussed above. Secondly, infection places additional stress on anabolic recovery processes and might increase the dietary requirement for zinc. Thirdly, a low-molecular-weight zinc-binding factor (ZBF) that facilitates zinc absorption has been indentified in pancreatic juice of experimental animals (32). Although most of our patients were taking pancreatic extracts to correct the pancreatic exocrine deficiency, the adequacy of ZBF in these preparations or in endogenous secretions is unknown. Lack of ZBF may be responsible for the observed malabsorption of zinc in cystic fibrosis (25).

CONCLUSION

To present study failed to confirm two findings previously reported for cystic fibrosis: 1) we failed to observe a depression in plasma zinc in growth-retarded patients; and 2) we did not encounter decreased taste thresholds. However, hair zinc and taste acuity data do suggest an incidence of zinc deficiency among individual patients in a population of juvenile cystic fibrosis attending a specialized outpatient clinic. Although zinc supplementation of cystic fibrosis patients has been reported previously (9), there was insufficient indication of *pre-existing* zinc deficiency in that population to raised expectation about dramatic effects. In the context of the present findings, the therapeutic implications of zinc administration to *zinc-deficient* patients with cystic fibrosis remain to be explored.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. Robert Rosenfield and Ms. Ann Eggleton for their assistance in studying the normal adolescent control subjects.

REFERENCES

1. SPROUL, A. and HUANG, N. Growth patterns in children with cystic fibrosis. J. Pediat., 65: 664-676, 1964.

2. SANDSTEAD, H.H., PRASAD, A.S., SCHULERT, A.R., FARID, Z., MIALE, A., BASSILLY, S. AND DARBY, W.J. Human zinc deficiency, endocrine manifestations and response to treatment. Am. J. Clin. Nutr., 20: 422-442, 1967.

3. HALSTED, J.A., RONAGHY, H.A., ABADI, P., HAGHSHENASS, M., AMIRHAKEMI, G.H., BARAKAT, R.M. and REINHOLD, J.G. Zinc deficiency in man. The Shiraz experiment. Am. J. Med., 53: 277-284, 1972.

4. PRASAD, A.S., SCHOOMAKER, E.B., ORTEGA, J., BREWER, G.J., OBERLEAS, D. and OELSHEGEL, F.J. Zinc deficiency in sickle cell anemia. Clin. Chem., 21: 582-586, 1975.

5. SOLOMONS, N.W., ROSENBERG, I.H., SANDSTEAD, H.H. AND VO-KHACTU, K.P. Zinc deficiency in Crohn's disease. Digestion, 16: 87-95, 1977.

6. McCLAIN, C., SOUTOR, C. AND ZIEVE, L. Zinc deficiency: A complication of Crohn's disease. Gastroenterology, 78: 272-279, 1980.

7. DODGE, J.A. and YASSA, J.G. Zinc deficiency syndrome in a British youth with cystic fibrosis. Br. Med. J., 1: 411-412, 1978.

8. HALSTED, J.A. and SMITH, J.C. Plasma zinc in health and disease. Lancet, 1: 322-324, 1970.

9. PALIN, D., UNDERWOOD, B.A. And DENNING, C.R. The effect of oral zinc supplementation on plasma levels of vitamin A and retimol-binding protein in cystic fibrosis. Am. J. Clin. Nutr., 32: 1253-1259, 1979.

10. HENKIN, R.I. Newer aspects of copper and zinc metabolism. In: Newer Trace Elements in Nutrition. W. Mertz and W.E. Cornatzer (eds.) Marcel Dekker, New York, 1971, p. 255.

11. HAMBIDGE, K.M., HAMBIDGE, C., JACOB, M. and BAUM, J.D. Low levels of zinc in hair, anorexia, poor growth, and hypogeusia in children. Pediat. Res., 6: 868-874, 1972.

12. HENKIN, R.I., SCHECHTER, P.J., HOYE, R. And MATTERN, C.F.T. Idiopathic hypogeusia with dysgeusia, hyposmia, and dysosmia: a new syndrome. J. Am. Med. Assoc., 217: 434-440, 1971.

13. ATKIN-THOR, E., GODDARD, B.W., O'NION, J., STEPHEN, R.L. and KOLFF, W.J. Hypogeusia and zinc depletion in chronic diaglysis patients. Am. J. Clin. Nutr., 31: 1948-1951, 1978.

14. WEISMANN, K., CHRISTENSEN, E. and DREYER, V. Zinc supplementation in alcoholic cirrhosis. Acta Med. Scand., 205: 361-366, 1979.

15. MAHJAN, S.K., PRASAD, A.S. LAMBUJON, J., ABBASI, A.A., BRIGGS, W.A. and McDONALS, F.D. Improvement of uremic hypogeusia by zinc: a double-blind study. Am. J. Clin. Nutr., 33: 1517-1521, 1980.

16. HENKIN, R.I. AND POWELL, G.F. Increased sensitivity of taste and smell in cystic fibrosis. Science, 138: 1107-1108, 1962.

17. WOTMAN, S., MANDEL, I.D., KHOTIM, S., THOMPSON, R.H., KUTSCHER, A.H., ZEGARELLI, E.V. and DENNING, C.R. Salt taste thresholds and cystic fibrosis. Am. J. Dis. Child., 108: 372-374, 1964

18. MANLAPAS, F.C., STEIN, A.A., PAGLIARA, A.S., APICELLI, A.A., PORTER, I.H., and PATTERSON, P.R. Phenylthiocarbamide taste sensitivity in cystic fibrosis. J. Pediat., 66: 8-11, 1965.

19. HERTZ, J., CAIN, W.S., BARTOSHUK, L.M. And DOLAN, T.F., Jr. Olfactory and taste sensitivity in children with cystic fibrosis. Physiol. Behav., 14: 89-94, 1975.

20. DESOR, J.A. and MALLER, O. Taste correlates in disease states: Cystic fibrosis. J. Pediat., 87: 93-96, 1975.

21. SOLOMONS, N.W., WAGONFELD, J.B., RIEGER, C., JACOB, R.A., BOLT, M., VANDER HORST, J., ROTHBERG, R. and SANDSTEAD, H.H. Some biochemical indices of nutrition in treated cystic fibrosis patients. Am. J. Clin. Nutr., 34: (in press).

22. JACOB, R.A. SANDSTEAD, H.H., SOLOMONS, N.W., RIEGER, C. and ROTHBERG, R. Zinc status and vitamin A transport in cystic fibrosis. Am. J. Clin. Nutr., 31: 638-644, 1978.

23. SINHA, S.N. and GABRIELI, E.R. Serum copper and zinc in various pathological conditions. Am. J. Clin. Pathol., 54: 570-577, 1970.

24. KLEVAY, L.M. Hair as a biopsy material. I. Assessment of zinc nutriture. Am. J. Clin. Nutr., 23: 284-289, 1970.

25. AGGETT, P.J., THORN, J.M., DELVES, H.T., HARRIES, J.T. and CLAYTON, B.E. Trace element malabsorption in exocrine pancreatic insufficiency, Monogr. Paediat., 10: 8-11, 1979.

26. KOPITO, L. and SHWACHMAN, H. Spectroscopic analysis of tissues from patients with cystic fibrosis and controls. Nature, 202: 501-502, 1964.

27. BRAND-AURABAN, A., KOPITO, L. and SHWACHMAN, H. Chemical analysis of some inorganic elements in cerumen from patients with cystic fibrosis. J. Invest. Dermatol., 58: 14-15, 1972.

28. SOLOMONS, N.W. On the assessment of zinc and copper nutriture in man. Am. J. Clin. Nutr., 32: 856-871, 1979.

29. PEKAREK, R.S. and BEISEL, W.R. Characterization of the endogenous mediator(s) of serum zinc and iron depression during infection and other stresses. Proc. Soc. Exp. Biol. Med., 138: 723-731, 1971.

30. BEISEL, W., PEKAREK, R.S. and WANNEMACHER, R.W., Jr. Homeostatic mechanisms affecting plasma zinc levels in acute stress. In: Trace Elements in Health and Disease. Vol 1. Zinc and copper. A.S. Prasad (ed.) Academic Press, New York, 1976, pp. 87-106.

31. SOLOMONS, N.W., ELSON, C.O., PEKAREK. R.S., SANDSTEAD, H.H. and ROSENBERG, I.H. Leukocytic endogenous mediator in Crohn's disease. Infect. Immun., 22: 637-639, 1978.

32. EVANS, G.W., GRACE, C.I. and VOTAVA, H.J. A proposed mechanism for zinc absorption in the rat. Am. J. Physiol. 228: 501-505, 1975.

NUTRITION RESEARCH, Vol. 1, pp. 25-31, 1981
0271-5317/81/010025-07$02.00/0 Printed in the USA.
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BREAST FEEDING, GROWTH AND MORBIDITY*

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ABSTRACT

The effect of exclusive breast feeding in the first few months of life on physical growth was studied prospectively in a group of 36 full-term healthy newborns. Growth failure was not observed in any infant until after the third month of life. The average growth curve for the entire group was between the 25th and 50th percentile at the end of the study period. However, faltering of growth as judged by weight at or below the tenth percentile of standard for age was seen in three (8%) infants at the age of four months, five (13%) at five months, eight (22%) at six months, nine (25%) at seven months, and twelve (33%) at eight months. Morbidity experience showed a slight but statistically higher frequency of respiratory infections and otitis in those infants who had shown faltered growth. Volume of milk intake was similar in the two groups. These observations suggest that a small proportion of exclusively breast-fed infants may not achieve adequate growth. In such infants, consideration should be given to supplementation after 4 months of age.

KEY WORDS: Breast feeding, infection, growth failure.

The unique physiochemical and immunological qualities of human milk are well established (1-7). In addition, the intimate physical contact between the mother and the infant enhances bonding and psychosocial interaction that is likely to have longterm consequences for mental health. These features have provided support to the general recommendation that breast milk should be the preferred exclusive source of food for the young infant. It is expected on physiological grounds, however, that at some time later in the first year of life, breast milk alone would not meet the infant's nutrient requirements and supplementary food would be needed. The exact age at which the provision of such dietary items is necessary is not agreed upon nor are there enough data on the incidence of growth failure and its clinical significance in exclusively breast-fed infants. In this paper, the growth and morbidity experience of a group of exclusively breast-fed babies are described.

MATERIAL AND METHODS

A group of 36 infants, 21 girls and 15 boys, fed exclusively on breast milk, were followed prospectively from birth to 9 months of age. The parents

*Presented in part at the International Symposium on Breast Feeding, Tel Aviv, Israel, February 25-28, 1980 and the Federation of American Societies for Experimental Biology, April 14-18, 1980.

were of middle socioeconomic status. Twenty-two mothers and 29 fathers had received university education. There were six first-born infants, 18 of birth order two, and 12 of birth order three.

Weight was measured accurately on a beam balance at monthly intervals, within three days of the birth date. The parents were contacted by telephone once in two weeks and morbidity data recorded. In all instances, the diagnosis of illness was confirmed on physical examination. Respiratory illness was suspected on the basis of cough, fever, abnormal feeding behaviour and corhyza for more than 24 hours. Physical signs included pharyngeal and nasal congestion, rhonchi or rales. Otitis was suspected on the basis of feeding problems, fretfulness and fever, and confirmed by the presence of dull, red or bulging tympanic membrane.

Breast milk intake was measured by standard test weighing technique immediately before and after feeding using Health-O-Meter scale (Continental Scale Corporation, Bridgeview, Illinois). Vitamin and mineral supplements were given to all the infants beyond 4 months of age.

Means of body weights were calculated for each month of life and plotted on growth charts prepared on the basis of the National Center for Health Statistics (NCHS) growth data (8). Differences in morbidity were analyzed by the X^2 test and in breast milk volumes by Student's t test.

RESULTS

The average growth curves for the entire group of boys and girls are shown in Figs. 1 and 2. The mean body weight was at or above the 50th percentile from

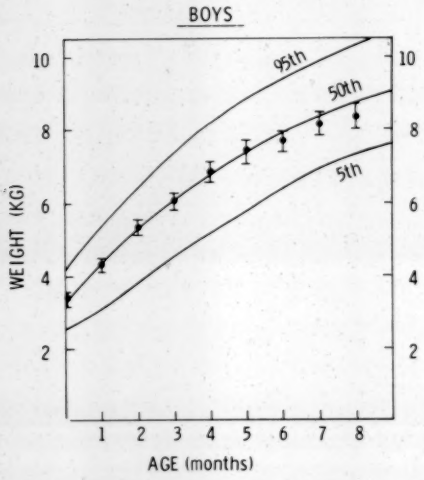

FIG. 1

Weight of boys being exclusively breast-fed. Data are shown as means ± S.D. and are superimposed on 95th, 50th, and 5th percentile of standards for age derived from the National Center for Health Statistics data.

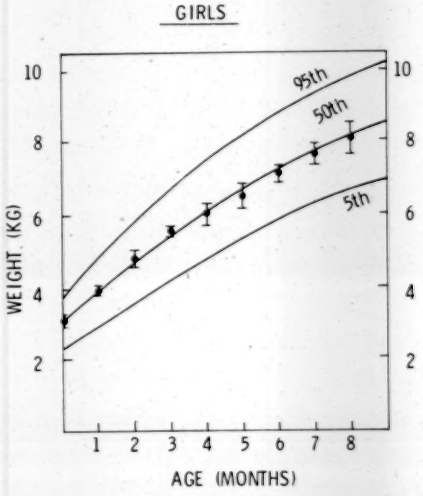

FIG. 2

Weight of girls being exclusively breast-fed. Data are shown as means ± S.D. and are superimposed on 95th, 50th, and 5th percentile of standards for age derived from the National Center for Health Statistics data.

birth to five months and between the 25th and 50th percentiles from 6 to 8 months. Weight fall-off was slightly more pronounced in boys. Growth faltering as judged by body weight below the tenth percentile is shown in Table 1. The

TABLE 1

Growth Failure in 36 Exclusively Breast-Fed Infants

Age (months)	Number Showing Growth Faltering (and percentage)
4	3 (8.3)
5	5 (13.6)
6	8 (22.2)
7	9 (25.0)
8	12 (33.3)

incidence of growth failure increased progressively from 8% at the age of 4 months to 33% at 8 months.

Twenty-four-hour breast milk intake is shown in Table 2. There was no

TABLE 2

Volume of Breast Milk Ingested in 24-Hours, (Mean ± S.E.M.)

Age (months)	BREAST MILK (ml)	
	Infants with Adequate Growth	Infants with Faltered Growth
3-4	793 ± 71	764 ± 85
4-5	856 ± 99	887 ± 83
5-6	925 ± 112	969 ± 101
6-7	872 ± 126	839 ± 96
7-8	815 ± 97	847 ± 103

significant difference between the total volume of milk ingested by infants

with adequate growth velocity and those exhibiting faltering of growth.

Morbidity experience is shown in Table 3. Both respiratory illness and

TABLE 3

Morbidity Experience Related to the Adequacy of Growth Velocity. Results are Expressed as Number of Episodes per 100 Child-Weeks.

	Adequate Growth	Faltered Growth*	P
Respiratory illness	2.89	7.20	< 0.01
Otitis	0.56	1.69	< 0.01

*Included in this group are infants who were ≤ 10th percentile of weight-for-age NCHS standards. The number of infants was 3 at the end of 4 months, 5 at 5 months, 8 at 6 months, 9 at 7 months and 12 at 8 months. The data were not broken down by sex because of the small numbers involved.

otitis occurred more often in infants with body weight at or below the 10th percentile of standard for age. None of the infants developed diarrhoea, skin infection, or conjunctivitis.

The birth weight of infants with growth faltering (3.36 ± 0.46 kg) was comparable with that of infants who maintained good growth (3.31 ± 0.53 kg). The birth length in the two groups was 49.6 ± 1.8 cm and 50.1 ± 2.1 cm respectively; the difference is not statistically significant. Birth order and interval since last pregnancy, if any, were comparable in the two groups.

DISCUSSION

The nutritional adequacy of breast milk from healthy, well-nourished mothers in a favorable environment as the only source of food for the majority of healthy infants up to 8 months of age was documented in this study. A small proportion, however, showed evidence of reduced growth velocity at and after four months. The prevalence of growth failure diagnosed on the basis of body weight at or below the 10th percentile of standard for age increased progressively from 8% in 4-month-old infants to 33% at the age of 8 months. The validity of NCHS growth curves for the largely formula-fed infants has been confirmed in our experience. However, the appropriateness of NCHS standards of growth can be debated since very few of the infants in that study were exclusively breast-fed. Nevertheless, weight less than the 10th centile of NCHS curves is likely to indicate true growth failure. This is also supported by morbidity experience (vide infra). In a recent study based on retrospective analysis, it was observed that exclusive breast feeding supported growth above the 25th centile up to the 9th or 10th month of life (9). The study cited is poorly presented and its conclusions unjustified. For instance, only average growth curves were presented, the 25th percentile is not an adequate cut-off point for adequate growth and thus it is not clear if some of the infants may have had growth failure.

Since the average volume of milk ingested was similar in the two groups of infants with adequate growth or faltered growth, the explanation for the inability to gain optimal weight must be in differences in requirements. There are very little data on the range of variation in the physiological energy needs of infants. It is not established whether an infant with a higher-than-average requirement will obtain more breast milk (10). The variability in the absolute volume of milk ingested is apparently not linked to differences in body size. In fact, in one investigation, milk intake per kg was negatively correlated with body weight (11). The theoretical and practical considerations of the nutritional adequacy of breast feeding have been reviewed recently (12).

Our estimates of daily intake of breast milk are generally comparable to those previously reported (13-15). Beyond three months of age, however, the amount of milk ingested in our study group was higher than Wallgren's data (15). The inherent difficulties in collecting this information and variations of intake in different 24-hour periods are recognized. Moreover, there may be a discrepancy between the actual volume of milk ingested and the "potential" of the mother to increase the output depending upon the vigor and duration of suckling and the energy requirements of the individual infant.

Infection-related morbidity was significantly higher in the group of infants who exhibited deceleration in growth velocity. There could be two alternative explanations for this finding. One, growth failure due to inadequate nutrient intake results in subtle impairment of immunocompetence and increased susceptibility to infection (16, 17). We have suggested that measurement of immunocompetence can be a useful functional index of nutritional status (18, 19). Thus higher morbidity in the small group of breast-fed infants who showed growth retardation may well be causally related to nutritional deficiency and impaired immunity. Two, infection itself could have produced growth faltering. Infectious illness is accompanied by a series of metabolic changes and decreased appetite, which may adversely influence weight gain (20, 21). However, this seems to be a less likely explanation in our study group, since in the majority of infants, growth failure was detected before clinical infections were observed. The existence of a subclinical infection in incubation producing growth failure in a few infants cannot be excluded, however, this is most unlikely. In a retrospective study (9), it was reported that about one-fourth of exclusively breast-fed infants had experienced "common" or "minor" illnesses such as otitis and colds and 3 out of 6 infants with bronchitis or meningitis showed decreased growth velocity corresponding to the illness.

In conclusion, out observations indicate that exclusive breast feeding even by well-nourished mothers in a favorable environment may not sustain optimal growth in a small proportion of infants beyond 4 months of age and that such growth faltering is associated with higher infectious morbidity, even though the types of illnesses could be considered as "minor". The data highlight the importance of careful monitoring of anthropometry in exclusively breast-fed infants so that the appropriate time to supplement breast milk in the small minority who would show deceleration of growth can be determined. It is not appropriate to base this decision on an arbitrary age cut-off. On the other hand, in underprivileged communities with poor sanitation, the option of supplemental intake has to be carefully weighed against the common chance of infection conveyed through the vehicle of contaminated food (22).

REFERENCES

1. Nutrition Committee of the Canadian Pediatric Society and the Committee on Nutrition, American Academy of Pediatrics: Breast-feeding. Pediatrics 59: 591-601, 1978.

2. Hambraeus, L., Forsum, E. and Lonnerdal, B. Nutritional aspects of breast milk versus cow's milk formula. In: Food and Immunology. Symposia of the Swedish Nutrition Foundation XII Stockholm Almqvist & Wiksell International, 116-124, 1977.

3. Cunningham, A.S. Morbidity in breast-fed and artifically-fed infants. J. Pediatr 725-729, 1977.

4. Mathew, D.J., Taylor, B., Normal, A.P., Turner, M.W. and Soothill, J.F. Prevention of eczema. Lancet i: 321-324, 1977.

5. Chandra, R.K. Immunological aspects of human milk. Nutr Rev 36: 265-272, 1978.

6. Chandra, R.K. Prospective studies of the effect of breast feeding on incidence of infection and allergy. Acta Paediatr Scand 68: 691-696, 1979.

7. Downham, M.A., Scott, P.S., Sims, D.G., Webb, J.K.G. and Gardner, P.S. Breast feeding protects against respiratory syncytial virus infections. Brit Med J ii: 274-276, 1976.

8. National Center for Health Statistics: NCHS growth curves for children birth to 18 years. United States. Department of Health, Education and Welfare, Publication (PHS) 78-1650, 1977.

9. Ahn, C.H. and MacLean, W.C. Growth of the exclusively breast-fed infant. Am J Clin Nutr 33: 183-192, 1980.

10. Evans, T.J. Growth and milk intake of normal infants. Arch Dis Childh 53: 749-51, 1978.

11. Ounsted, M. and Sleigh, R. The infant's self-regulation of food intake and weight gain. Lancet i: 1393-1395, 1975.

12. Waterlow, J.C. and Thomas, A.M. Observations on the adequacy of breast-feeding. Lancet ii: 238, 1979.

13. Jelliffee, D.B. and Jelliffee, E.P. The volume and composition of human milk in poorly nourished communities. A review. Am J Clin Nutr 31: 492-515, 1978.

14. Lonnerdal, B., Forsun, E. and Hambraeus, L. A longitudinal study of the protein, nitrogen and lactose contents of human milk from Swedish well-nourished mothers. Am J Clin Nutr 29: 1127-33, 1976.

15. Wallgren, A. Breast milk consumption of healthy full-term infants. Acta Paediatr Scand 32: 778-90, 1944.

16. Chandra, R.K. Immunology of Nutritional Disorders. Arnold, London, 1980.

17. Chandra, R.K. and Newberne, P.M. Nutrition, immunity and infection: Mechanisms of interactions. Plenum, New York, 1977.

18. Chandra, R.K. Immunocompetence is a functional index of nutritional status. Brit Med Bull (in press) 1981.

19. Chandra, R.K. and Scrimshaw, N.S. Immunocompetence in nutritional assessment. Am J Clin Nutr (in press) 1980.

20. Martorell, R. Illness and Incremental Growth in Young Guatemalan Children. Doctoral Dissertation, University of Washington 1975.

21. Mata, L.J., Kromal, R.A., Urrutia, J.J. and Garcia, B. Effect of infection on food intake and the nutrition state: perspectives as viewed from the village. Am J Clin Nutr 30: 1215-27, 1977.

22. Rowland, M.G.M., Barrell, R.A.W. and Whitehead, R.G. Bacterial contamination in traditional Gambian weaning foods. Lancet i: 136-138, 1978.

NUTRITION RESEARCH, Vol. 1, pp. 33-46, 1981
0271-5317/81/010033-14$02.00/0 Printed in the USA.
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INFECTIONS ASSOCIATED WITH SEVERE PROTEIN-CALORIE MALNUTRITION IN HOSPITALIZED INFANTS AND CHILDREN

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ABSTRACT

One hundred Bangladeshi children admitted to hospital for treatment of severe protein-calorie malnutrition were systematically evaluated for the presence of infections. Ninety percent of children had some evidence of systemic infection at the time of admission and 75% had pneumonia, bacteruria, diarrhea in association with a known enteric pathogen, bacteremia, meningitis, or more than one of these major infections. Forty-nine percent of patients had pneumonia, including 14% of admissions with clinical evidence of pulmonary tuberculosis. Forty-three percent of admissions had diarrhea and 40% had evidence of enteric infections, most commonly shigellae or rotavirus. Bacteruria occurred in 30% of admissions, but bacteremia was identified in only 2% of patients initially. The prevalence of intestinal parasites increased with age, both among inpatients and comparison subjects with less severe grades of malnutrition. There did not appear to be important differences in the parasite loads or prevalences between the 2 groups.

Twenty-one inpatients died; deaths were more common in younger children. The cause of death was most frequently related to infections. The identification and appropriate treatment of infections must be considered a major component of the rehabilitation of severely malnourished children.

KEY WORDS: Protein-calorie malnutrition, pneumonia, bacteruria, enteric infections, parasites.

INTRODUCTION

The association between malnutrition and infectious diseases has been long recognized and widely accepted (1). However, surprisingly few clinical studies have attempted to describe the types of infections identified among severely malnourished children and to define the etiologic agents responsible for those infections. To provide more information on the range of infections associated with severe protein-calorie malnutrition (PCM) this communication reports all such illnesses that were diagnosed in one hundred

patients hospitalized for the treatment of severe PCM at the Children's Nutrition Unit in Dacca, Bangladesh.

METHODS

Patient selection. All children attending the Children's Nutrition Unit's outpatient clinic were eligible for admission to the inpatient ward if they fulfilled any of the study's criteria for severe PCM. The children were usually brought to the clinic because of some acute problem (most commonly fever or diarrhea) or chronic failure to thrive. Some patients were referred from other treatment centers specifically because of malnutrition. All clinic attenders were weighed, measured and classified for nutritional status as compared to the NCHS/CDC reference population (2). From limited Bangladeshi village growth data (unpublished) we estimated the median expected weight for age to be approximately 69% of the NCHS/CDC median, and the median expected weight for length to be approximately 90% of the reference median. In order to restrict hospital admission to those children who were most severely undernourished, we selected only those who were less than 60% of the local median weight for age (i.e., 42% of the NCHS/CDC median) or less than 70% of the local median weight for length (i.e., 63% of the NCHS/CDC median). We also admitted to the inpatient facilities those children with edema, hypoproteinemia, hair, skin and mental changes, a constellation of clinical signs that were assumed to indicate kwashiorkor. Males and females up to 10 years of age were eligible for hospital admission; the inpatient study took place from January through April, 1976 and included one hundred consecutive admissions. Children with less severe grades of undernutrition were treated in the hospital's day care feeding program or outpatient clinic.

Nutritional diagnoses were based on the McLaren criteria for marasmus, kwashiorkor, and marasmic-kwashiorkor (3). Patients with severe vitamin A deficiency were classified by primary physical signs, as discussed in the joint WHO/USAID Report of vitamin A deficiency and xerophthalmia (4).

For the sake of comparison, a sampling of outpatients with less severe degrees of undernutrition were evaluated for selected infections, namely enteric infections and intestinal parasites. Outpatient subjects included an unselected sample of acutely ill children who reported to the Children's Nutrition Unit clinic between April and December, 1976 but did not meet criteria for hospital admission. Village comparison subjects were children from 1 to 6 years of age who participated in an unrelated, but concurrent field study; they were identified from a register of patients previously treated for acute diarrhea at the field station of the International Center for Diarrhoel Disease Research (ICDDR).

Diagnostic studies. Rectal temperatures were recorded every 4 hours during the first week of hospitalization. A temperature greater than 38.1° C was considered fever and less than 35.6° C was defined as hypothermia. Laboratory studies at the time of admission included hematocrit, total and differential white blood cell count, urinalysis, microscopic examination of the stool for red blood cells, pus, ova, and parasites, and total plasma protein by refractometer. Intermediate strength tuberculin skin tests were applied. Most children were transported to the ICDDR for a single AP chest radiograph within the first 48 hours of admission. The x-rays were evaluated for infiltrates, pleural effusions, and hilar adenopathy. Radiographic patterns that were considered suggestive of pulmonary tuberculosis included diffuse miliary disease, hilar adenopathy with infiltrates, and infiltrates with pleural effusions. Pulmonary tuberculosis was diagnosed when a suggestive radiographic pattern was associated with a clinical picture such as positive family history, positive tuberculin skin test, unexplained fevers in the hospital, or growth failure in hospital despite adequate energy intake and absence of clinical signs of malabsorption. Reliable facilities for culturing of mycobacteria were unavailable.

Throat swabs, blood, stool and urine specimens were routinely taken for aerobic bacteriologic cultures. Urine was obtained by percutaneous suprapubic bladder aspiration or collection of a freshly voided specimen in the event of a "dry tap". The diagnosis of bacteruria was based on the finding of any bacterial growth in urine obtained by suprapubic tap or greater than 100,000 colonies per ml of urine obtained from a freshly voided specimen. The clinical diagnosis of diarrhea was based on the finding of more than 4 loose or watery stools per day in children over 12 months of age, or more than 6 loose or watery stools per day in infants less than 12 months. Children with less than the critical number of loose stools, but with mucus and blood in a loose stool on gross or microscopic exam, were included in the diarrhea group.

With the exceptions noted below all bacteriologic specimens were processed by the routine bacteriology laboratory of the ICDDR according to standard procedures (5). Ten individual lactose-fermenting colonies and a pool of 10 colonies were picked to test for production of heat-labile enterotoxin by the Y_1 adrenal cell culture method (6), and to test their ability to invade the guinea pig conjunctivae (7). Rotavirus infections were diagnosed when the viral antigen was both identified in feces by the ELISA technique and then confirmed by blocking tests (8), or when complement fixation tests of paired sera demonstrated a fourfold rise of antibodies to either O or NCDV antigens (9). At the time of the clinical studies facilities were not available for the identification of heat-stable E. coli enterotoxin, Yersinia enterocolitica, or Campylobacter jejuni.

Separate portions of fresh fecal specimens were suspended in saline or concentrated in formalin-ether. Specimens prepared in both ways were then examined microscopically for ova and parasites (10). Another portion of the original sample was placed in merthiolate-iodine-formalin, and a 2 mg sample of the emulsification was later examined in duplicate for total ova counts as described by Dunn (11). Children with mean ova counts greater than 100 for ascaris and Trichuris or greater than 10 for hookworms were defined as having heavy infections. Serum specimens were examined for anti-amoeba antibody titers by the tanned red cell hemagglutination technique using lysed axenic amoebae as the antigen (12). Serum titers greater than 1:128 were considered positive.

The treatment of severe PCM has been discussed elsewhere by us and by others (13,14) and is beyond the scope of this communication. Briefly, fluid and electrolyte therapy, an aggressive search for and treatment of infections, and dietary rehabilitation were the mainstays of our therapeutic regimen.

RESULTS

A summary of the patients' nutrition-related findings is presented in Table I; selected nutritional parameters of a group of Bangladeshi village children are listed for comparison. Two inpatients proved to have primary renal disease as the cause of their edema; their clinical data are not included in any of the tabulations.

Twenty-three inpatients had fever during the first 24 hours in the hospital and a total of 43 patients were febrile during the first week. Only one child was hypothermic. Of the 73 patients with documented major infections, 28 (38%) were never febrile or hypothermic. Fifty-five percent of the malnourished children had total white counts greater than 15,000 and none had a count less than 5,000. Thirty-two percent of children with major infections had no elevation in either the total white count (>15,000) or neutrophil count (>7,500) and eight percent of children with major infections had neither leukocytosis nor fever.

Table 1. Clinical characteristics of inpatients with severe protein-calorie malnutrition, outpatients, and village comparison subjects.

	(n)	Age[a] (mos)	Sex %M	%F	Wt (kg)	L (cm)	% expect wt/age[a]	% expect L/age[a]	% expect wt/L[a]	Plasma-Protein (g/dl)	HCT (%)	Subjects with Xeroph-thalmia No.	(%)	Night blindness No.	(%)	Angular Stomatitis No.	(%)
Inpatients																	
Marasmus	56	13[b] (12-18)	50	50	4.43[c] ±2.12	65.0[c] ±11.8	40.5[c] ±8.6	80.5[c] ±7.2	63.1[c] ±7.0	6.6[c] ±1.0	30.5[c] ±6.1	9	(16)	1	(2)	2	(4)
Marasmic-Kwashiorkor	28	40 (30-60)	46	54	7.43 ±3.00	80.0 ±14.0	46.7 ±10.7	81.0 ±7.5	67.4 ±10.0	5.0 ±0.8	24.4 ±6.8	9	(32)	2	(7)	8	(28)
Kwashiorkor	14	25 (14-42)	57	43	7.25 ±3.16	74.4 ±14.4	53.0 ±9.1	82.2 ±4.7	76.4 ±8.8	4.0 ±0.6	25.2 ±6.4	5	(36)	1	(7)	5	(36)
All Inpatients	98	20 (18-30)	50	50	5.70 ±2.92	70.7 ±14.4	43.8 ±10.0	80.9 ±7.0	66.3 ±9.4	5.8 ±1.4	28.0 ±6.9	23	(23)	4	(4)	15	(15)
Outpatients	146	24 (22-32)	60	40	8.8 ±3.4	79.8 ±16.4	68.5 ±11.8	89.0 ±8.0	84.3 ±10.3	7.3 ±0.8	34.1 ±4.1	NA	-	NA	-	NA	-
Village Comparison Subjects	94	32 (29-44)	65	35	10.06 ±2.54	82.3 ±10.0	67.2 ±10.5	85.6 ±5.6	86.1 ±8.1	7.8 ±0.6	35.6 ±3.4	0	(0)	3	(3)	2	(2)

[a] Age is not known with certainty in Bangladesh. These data must be interpreted as best approximations based on family histories and "local events" calendars. Anthropometrics were compared to CDC/NCHS reference data (2).

[b] Median (95% confidence limits)

[c] Mean ±1 S.D.

A summary of all major infections is listed in Table II in relation to the categories of PCM with which the infections appeared. A child was considered to have a major infection if an enteric pathogen was identified from a subject with diarrhea, if pneumonia was diagnosed by radiograph, or if bacteruria or bacteremia was present.

Forty-nine percent of malnourished children had radiologic evidence of pulmonary infections. Of these, 60% had pulmonary infiltrates that involved more than one lung segment; many patients had small infiltrates in numerous segments. Fourteen percent of the inpatients were felt to have evidence of pulmonary tuberculosis because of a suggestive x-ray picture and consistent clinical findings. Three of the 14 patients had radiologic evidence of miliary tuberculosis and one patient had a pleural effusion and spontaneous pnemothorax. Only one-third of the patients with clinically diagnosed tuberculosis responded to an intermediate strength tuberculin skin test with greater than 10 mm of induration at the time of admission. Of the 21 patients who died, eight had abnormal chest x-rays of which five were suggestive of pulmonary tuberculosis.

Thirty percent of malnourished patients for whom urine culture results are available had bacteruria. Twenty-eight percent of 72 bladder aspirations and 37% of 19 voided specimens gave positive cultures. Eleven positive cultures were obtained from males. E. coli was recovered from 26 of the 27 positive cultures; pseudomonas was found in one. Two of the patients with urinary tract infections died on the second hospital day. One of these patients had a different organism in the blood culture from that in the urine; the other had negative blood cultures and died with respiratory distress. A third patient died on the twentieth day of hospitalization with septicemia from an unrelated organism. Urinalysis results were available from twenty bacteruric patients prior to antibiotic therapy. Only twelve (60%) of those patients had pyuria (more than 5 WBC's per high powered microscopic field).

Forty-three percent of malnourished patients had diarrhea during the first 48 hours in the hospital; two patients (2%) had both blood and mucous on direct stool examination and twenty-eight patients (29%) had pus cells on microscopic examination. Of the ninety-seven admission stool culture results that were available 33 specimens were positive, including three with two pathogens. Rotavirus infections were diagnosed only on the basis of serological findings in an additional 7 patients. The various enteric pathogens and their frequency of recovery are listed in Table III. Rotavirus and shigellae accounted for most of the infections. As expected, the prevalence of rotavirus infections was significantly greater in children 24 months of age or less (31%) than in those more than 24 months (13%), $p<0.05$. Of the 10 children who had rotavirus identified on the initial stool examination and who had results available from a second stool exam two to three weeks after admission, only one was still excreting rotavirus. Enterotoxigenic E. coli was recovered from only two inpatients; entero-invasive E. coli was not found in any of the 93 subjects studied. Just over half the cases of diarrhea were associated with known enteric pathogens; pathogens were found much more frequently among inpatients than outpatients ($p<0.001$). Among inpatients who died, 10 had diarrhea initially. However, diarrhea was still present at the time of death in only five children, including one with rotavirus.

Although admission blood cultures were obtained from all patients, only one specimen, which was drawn from a patient with osteomyelitis, was positive. Another patient who had fever, headache and splenomegaly on admission had a negative initial blood culture; but Salmonella typhi was recovered subsequently from his blood. Additional positive blood cultures found later during the course of hospitalization are not included in the present tabulations of admission findings.

Table II. Number of inpatients with major infections present at time of admission[a].

	Pneumonia (including presumed Tuberculosis)	Bacteriuria	Diarrhea (clinically defined)	Enteric Infection[b]	Bacteremia[c]	Meningitis	Patients with one or more major infections
Marasmus (n=56)	25/54[a] (46%)	9/51[d] (18%)	25/56 (45%)	23/55 (42%)	1/56 (2%)	1 (2%)	40/56 (71%)
Kwashiorkor and Marasmic-Kwashiorkor (n=42)	22/41 (54%)	18/40[d] (45%)	17/42 (40%)	15/41 (37%)	1/42 (2%)	0	33/42 (78%)
All Inpatients (n=98)	47/95 (49%)	27/91 (30%)	42/98 (43%)	38/96 (40%)	2/98 (2%)	1 (1%)	73/98 (75%)

[a] Culture specimens and appropriate x-rays were not always available at the time of admission. Denominators refer to number of patients for whom appropriate diagnostic studies were available. Figures for meningitis and for patients with at least one major infection, however, refer to total number of inpatients in each category.

[b] Defined as isolation of Vibrio Cholera, Salmonella, Shigella, enterotoxigenic E. coli (heat-labile toxin), and/or rotavirus from stool or rotavirus seroconversion.

[c] One patient with osteomyelitis; one patient with typhoid fever.

[d] Edematous vs non-edematous significantly different by chi-square, $p<0.025$.

Table III. Pathogens recovered from initial stool specimens and paired rotavirus serology results from inpatients with severe PCM and outpatient comparison subjects

	Shigella[a]	Salmonella (Group 1)	ETEC-LT[b]	Rotavirus: Stool only	Rotavirus: Sero. Conv. only	Rotavirus: Stool and Serum pos.	Rotavirus: Total	Subjects with one or more Pathogens (Total No.)
Inpatients								
with diarrhea (n=42)	13[c]/42 (31%)	1[c]/42 (2%)	1/33 (3%)	4/38 (10%)	5[c]/25 (25%)	4[c]/25 (16%)	13/38 (34%)	24/42 (57%)
without diarrhea (n=56)	5/54 (9%)	0/54 (-)	1/38 (3%)	4/53 (8%)	2/30 (7%)	2/30 (7%)	8/53 (15%)	14/55 (25%)
Significance of χ^2 diarrhea vs no diarrhea	p<0.01	N.S.	N.S.	N.S.	N.S.	N.S.	p<0.05	p<0.005
All Inpatients (n=98)	18/96 (19%)	1/96 (1%)	2/71 (3%)	8/91 (9%)	7/55 (13%)	6/55 (11%)	21/91 (23%)	38/96 (40%)
All Outpatients (n=146)	2/106 (2%)	0/106 (-)	-	2/52 (4%)	1/26 (4%)	0/2 (-)	3/80 (4%)	5/124 (4%)
Significance of χ^2 All inpatients vs all outpatients	p<0.001	N.S.	-	N.S.	N.S.	N.S.	p<0.001	p<0.001

[a] Inpatients' Shigella infections included flexneri (12), sonnei (3), boydii (2), and shiga (1); one subject had both boydii and dysenteriae, type 3-10.

[b] Enterotoxigenic E. coli (heat-labile toxin).

[c] Three inpatients had both Shigella flexneri and rotavirus infection, one had both Salmonella type E and rotavirus infection.

A variety of minor infections was also diagnosed at the time of initial examination. Thirteen children had acute or chronic otitis media, eleven had pyodermas, eight had oral moniliasis, and one had a dental abscess. Culture results from oropharyngeal swabs were available from all patients. Beta-hemolytic streptococci were found in eleven specimens (11%), but were not grouped. Interestingly, 85% of cultures from the pharyngeal swabs obtained at admission yielded a variety of gram negative bacilli.

The prevalences of specific parasitic infections among inpatients and outpatients are shown graphically in Figure 1. There was an age-related trend for the presence of all parasites. Outpatients had a significantly higher prevalence rate of ascariasis and trichuriasis than inpatients in some age groups, but inpatients in the older age groups had higher rates of hookworm and strongyloides infections. The prevalences of heavy worm burdens, as estimated from ova counts, are noted for Ascaris lumbricoides, hookworms, and Trichuris trichuria in the same figure. There were no statistically significant differences in the worm loads between groups. No children excreted hematophagous trophozoites of E. histolytica although two were passing cysts. Only one child had a positive serum titer for anti-amoeba antibodies. Among inpatients there were no clear-cut associations between the nutritional diagnostic category and specific parasitic infections. There were no differences in anthropometrics or total protein levels between inpatients with and without hookworms and trichuriasis and between those with and without heavy ascaris infections. Inpatients with hookworm infections had a lower mean hematocrit level (22.5±6.9) than those without hookworms (30.0±5.8, $p<0.001$). Outpatients had the same mean hematocrit levels regardless of hookworm status, but no heavy infections were seen among that group.

Twenty-one of the inpatients died during the course of hospitalization; six during the first 48 hours in hospital and a total of eleven during the first week. Survivors and non-survivors were generally similar with regard to nutritional diagnostic category and anthropometric status, but the non-survivors tended to be younger (median ages 12 vs 25 months). Nine patients aged 2-6 months died, giving an age-specific case fatality rate of 56% for that group.

In seven patients there were positive blood cultures either prior to or immediately after death; post-mortem percutaneous lung aspirates were positive for bacteria in an additional two patients, one of whom died with respiratory failure. The organisms recovered, frequency and site of recovery, and ages of the respective patients are listed in Table IV. Among the remaining non-survivors, respiratory failure secondary to severe pulmonary tuberculosis was felt to be the cause of death in three, whose ages were 15, 35 and 40 months; and one child died with meningitis of unknown etiology. The cause of death in the remaining eight was uncertain. Four of these patients (ages 5, 14, 18 and 68 months) had severe diarrhea of unknown etiology at the time of death, one (age 4 months) had diarrhea associated with rotavirus, one had unexplained respiratory distress and two had presumed tuberculosis.

DISCUSSION

The description of the clinical syndrome of severe PCM and its complications varies both according to geographic location and to the diagnostic criteria used to define the study population (15,16). In the present study acceptability for admission to the inpatient facility was determined by specific clinical and anthropometric indicators of nutritional status, regardless of the presence or absence of infection.

Major infections were documented initially in 75% of hospital admissions, leukocytosis or fever during the first hospital day was recorded in an additional 14% of patients. Thus, it is probable that approximately 90% of

PREVALENCE OF INTESTINAL PARASITES AMONG INPATIENTS WITH SEVERE PCM AND OUTPATIENTS

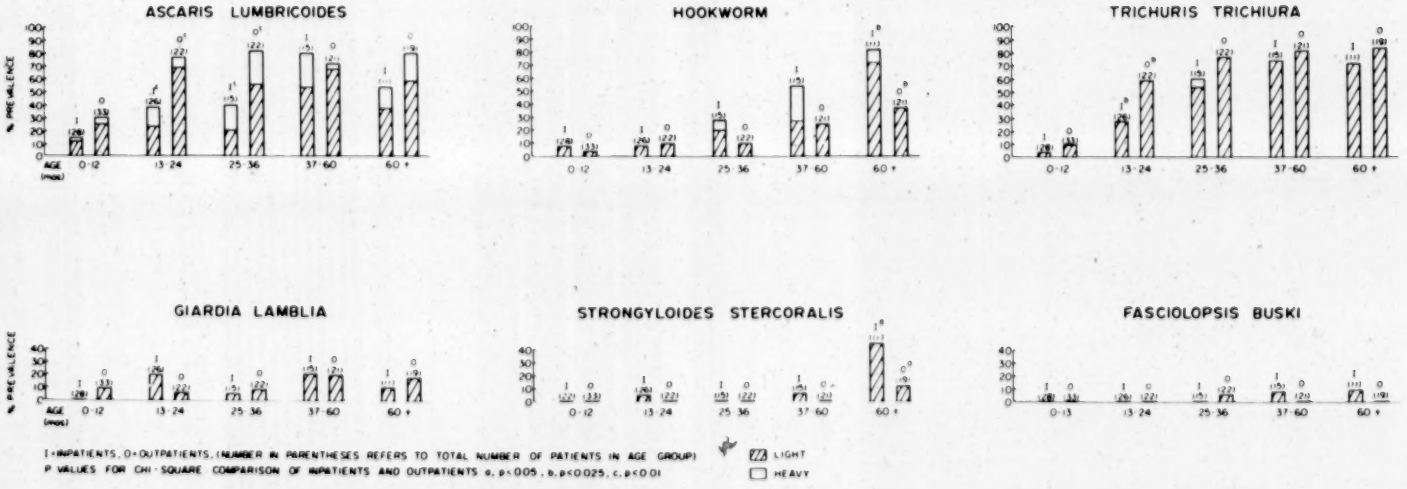

FIG. 1

children had systemic infections on the day of hospital admission. Common sites of infections included the respiratory, urinary, and gastrointestinal tracts; other superficial infections were also frequent.

TABLE IV
Bacteria Recovered in Pre- or Post-Mortem Blood and Post-Mortem Lung-Aspirate Cultures from Non-Survivors*

Organisms	Site and Frequency of Recovery	Ages of Patients From Whom Organisms Were Recovered (Months)	
		Blood	Lung
Pseudomonas Sp.	Blood (4), Lung (2)	2,4,5,26	5,15
E. coli	Blood (1), Lung (1)	2	5
Proteus Sp.	Blood (1)	5	-
Klebsiella pneumoniae	Lung (1)	-	5
Staphylococcus aureus	Blood (1)	24	-

* Total of 9 of 21 non-survivors with positive cultures. Seven children had positive blood cultures; two children had positive lung-aspirate cultures.

Pneumonia was the most commonly diagnosed infection in our patients, and associated respiratory failure was an important cause of mortality. Unfortunately, we were unable to determine the organism responsible for pneumonia in most cases. Pulmonary tuberculosis was diagnosed frequently and accounted for approximately 30% of pulmonary infections. Since unusual pulmonary pathogens have been recovered from malnourished patients and from other children with impaired host resistance (17,18) it seems that an investigation of etiological agents responsible for pulmonary infections in malnourished children would be a useful project for a center equipped to undertake such studies. The higher prevalence of pneumonia in our patients as compared to previous studies (19, 20,21) may be partially explained by the high rates of tuberculosis. An autopsy study of severely malnourished African children did note a 45% incidence of bronchopneumonia on pathological examination, underscoring the frequency of pneumonia in children who die (22).

Bacteruria was identified in 30% of inpatients, a rate similar to those reported by others (20,23). Although it appears that bacteruria occurred more commonly in edematous children (Table II), we have previously shown that bacteruria is associated with xerophthalmia and not hypoproteinemia (24). In the present series xerophthalmia was more common among edematous children ($p<0.002$).

Diarrhea was not found as frequently in our patients as in other series (25,26). However, we included only those cases of diarrhea that occurred within the first three hospital days, a time when malabsorptive causes of diarrhea are minimized by relatively low dietary intakes. On the other hand, enteric pathogens were isolated more commonly than others have reported (20,21, 26), perhaps because we plated the fecal specimens immediately and because we also searched for the more recently recognized pathogens, enterotoxigenic E. coli and rotavirus.

Shigellae and rotavirus were the agents most often associated with diarrhea in the malnourished subjects. However, the number of isolations of shigellae increased at the Cholera Research Laboratories as a whole during the period from 1973-1977, coincident with a presumed nation-wide epidemic of shigellosis (27). Therefore, the shigellae rates reported herein may not indicate a constant pattern for malnourished Bangladeshi children. Although rotavirus is now recognized as a cause of chronic diarrhea in immunosuppressed hosts (28), excretion of the virus for more than three weeks was found in only

one of the malnourished children. Heat-labile E. coli enterotoxin was identified in only two of 78 initial stool specimens. Since E. coli that produce only heat-stable toxin may be responsible for as much as 50% of the infections caused by enterotoxigenic E. coli (29), slightly more E. coli infections might have been diagnosed if we had been able to identify the heat-stable toxin.

Surprisingly few of the patients' initial blood cultures were positive. Although the rate of bacteremia would be increased by inclusion of positive cultures obtained later during the course of hospitalization, it would still not be as high as the rates (11 to 23%) reported by other workers (20,21). The high prevalence of gram negative bacilli isolated from oropharyngeal swabs is disconcerting in view of the high rates of gram negative bacteremia and pneumonia reported herein and elsewhere (21). It is likely that during the course of hospitalization children become infected with their own indigenous flora.

Although intestinal malabsorption of specific nutrients has been associated with some enteric parasites (30,31), the relationship between nutritional status and parasite infections is not well defined (32). In the present study there were no significant differences between the parasite loads of the malnourished inpatients and the better nourished outpatients, but the prevalence rates did vary between groups. Although the differences in age-specific prevalence rates achieved statistical significance for some parasites, the biologic importance of the findings is uncertain since the higher rates were not consistently observed in one or the other patient group. The lower mean hematocrit level of inpatients infected with hookworm was not unexpected in that hookworm is known to cause anemia to a degree proportional to the parasite load (33). The outpatients, whose hookworm loads were relatively light, did not demonstrate more severe anemia in relation to the parasite infection. Protozoal infections were relatively uncommon. Evidence for significant amoeba infections was not found except in one child with elevated antibody titers. Giardiasis was equally prevalent in inpatients and outpatients and was not significantly associated with diarrhea.

The high mortality rate among these patients is comparable to results reported from other hospitals with similar patient populations (25,34) and demonstrates the difficulty in successfully rehabilitating children with severe PCM. Although the cause of death was uncertain for many of the patients, overwhelming infections seemed directly responsible for the majority of deaths. The importance of infections has been demonstrated in other clinical (21,34) and autopsy reports (35,36).

Successful treatment of infections in malnourished children is complicated by the fact that the usual clinical indicators of infection may not be apparent. Our study confirms previous reports that malnourished children may fail to develop fever or leukocytosis despite a major infection (20). Similarly, pyuria is a poor indicator of urinary infections. Therefore, we believe that a careful search for infection must be undertaken in all severely malnourished children presenting themselves for therapeutic attention. In situations where diagnostic facilities are not available, antibiotic therapy should be considered while nutritional rehabilitation is initiated. Since organisms resistant to common antibiotics are frequently isolated from children dying with malnutrition, drugs active against gram negative bacilli, including enteric flora and pseudomonas species, should be available.

The present study did not attempt to elucidate the mechanisms of interaction between malnutrition and infectious diseases. Several authors have previously reviewed the various effects of malnutrition on immune responses, and postulate that immunosuppression may explain the frequency and severity of infections seen in malnourished individuals (1,37). Conversely, it has been amply shown that infectious diseases interfere with normal nutrition and growth (1,38). Tuberculosis and urinary tract infections for example, can

cause growth failure even in situations where the availability of food is not a problem. It is probable that the severe malnutrition observed in at least some of our patients was, in fact, secondary to their chronic infections. Regardless of the causal interactions, it is apparent that most cases of severe protein calorie malnutrition are complicated by a variety of infections; the identification and appropriate treatment of such infections must be considered a major component of the rehabilitation of severely malnourished children.

REFERENCES

1. SCRIMSHAW, N.S., TAYLOR, C.E., and GORDON, J.E. Interactions of nutrition and infection. W.H.O. Monograph Series No. 57, Geneva, 1968.

2. NCHS growth curves for children birth-18 years, United States. DHEW Publication No. (PHS) 78-1650, National Center for Health Statistics, U.S. Department of HEW, Hyattsville, 1977.

3. MCLAREN, D.S., PELLETT, P.L., and READ, W.W.C. A simple scoring system for classifying the severe forms of protein-calorie malnutrition of early childhood. Lancet, 1: 533-535, 1967.

4. WHO/USAID. Vitamin A deficiency and xerophthalmia. W.H.O. Technical Report Series No. 590, Geneva, 1975.

5. CRUICKSHANK, R., DUGUID, J.P., MARMION, B.P. and SWAIN, R. (eds): Medical Microbiology, II, 12th Edition, Churchill Livingstone, Edinburgh, 1975.

6. SACK, D.A., and SACK, R.B. Test for enterotoxigenic Escherichia coli using Y_1 adrenal cells in miniculture. Infect. Immun. 11: 334-336, 1975.

7. SERENY, G. Experimental shigella conjunctivitis---a preliminary report. Acta Microbiol. Acad. Sci. Hung. 2: 293-298, 1955.

8. KAPIKIAN, A.Z., YOLKEN, R.H., GREENBERG, H.B., WYATT, R.G., KALICA, A.R., CHANOCK, R.M. and KIM, H.W. Gastroenteritis viruses. In: Diagnostic Techniques - Viral, Rickettsial and Chlamydial Infections. E.H. Lennette & N. Schmidt (eds). American Public Health Association, Washington, 1979, pp. 927-995.

9. KAPIKIAN, A.Z., CLIVE, W.L., KIM, H.W., KALICA, A.R., WYATT, R.G., VANKIRK, D.H., CHANOCK, R.M., AMEX, H.D., and VAUGHN, A.L. Antigenic relationships among five reovirus-like (RVL) agents by complement fixation (CF) and development of new substitute CF antigens for the human RVL agent of infantile gastroenteritis. Proc. Soc. Exp. Biol. Med. 152: 535-539, 1976.

10. HUNTER, G.W., FRYE, W.W. and SWARTZWELDER, C.J. A manual of tropical medicine. W. B. Saunders Co., Philadelphia, 1960.

11. DUNN, F.L. The T.I.F. direct smear as an epidemiological tool. Bull. W.H.O. 37: 439-449, 1968.

12. KESSEL, J.F., LEWIS, W.P., MOLINA-PASQUALE, C. and TURNER, J.A. Indirect hemagglutination and complement fixation tests in amoebiasis. Am. J. Trop. Med. Hyg. 14: 540-550, 1965.

13. BROWN, K.H. The treatment of severe malnutrition in hospitalized Bangladeshi children. Bangladesh Paed. 3: 1-6, 1976.

14. SUSKIND, R. The inpatient and outpatient treatment of the child with severe protein-calorie malnutrition. In: Protein-Calorie-Malnutrition. R.E. Olson (ed). Academic Press, New York, 1975, pp. 404-414.

15. GARROW, J.S. and PIKE, M.C. The short-term prognosis of severe primary infantile malnutrition. Nutr. 21: 155-165, 1967.

16. MCLAREN, D.S., SHIRAJIAN, E., LOSHKAJIAN, H. and SHADAREVIAN, S. Short-term prognosis in protein calorie malnutrition. Am. J. Clin. Nutr. 22: 863-870, 1969.

17. HUGHES, W.T., PRICE, R.A., SISKO, F., HAVRON, W.S., KAFATOS, A.G., SCHONLAND, M., and SMYTHE, P.M. Protein-calorie malnutrition. A host determinant for Pneumocystis carinii. Am. J. Dis. Child. 128: 44-52, 1974.

18. HUGHES, W.T., FELDMAN, S. and COX, F. Infectious diseases in children with cancer. Ped. Clin. N. Am. 21: 583-615, 1974.

19. LANGUNDOYE, S.B. and REDDY, S. Chest x-ray changes in Kwashiorkor. J. Trop. Peds. 16: 124-129, 1970.

20. MOREHEAD, C.D., MOREHEAD, M., ALLEN, D.M., and OLSON, R.E. Bacterial infections in malnourished children. J. Trop. Ped. & Environ. Child Health 20: 141-147, 1974.

21. PHILLIPS, I. and WHARTON, B. Acute bacterial infection in Kwashiorkor and Marasmus. Brit. Med. J. 1: 407-413, 1968.

22. BROWN, R.E. Paediatric post mortem survey in Uganda. Trop. Geog. Med. 17: 289-300, 1965.

23. BUCHANAN, N., FAIRBURN, J.A., SCHMAMAN, A. and VOS, G.H. Urinary tract infection and secretory urinary IgA in malnutrition. S.A. Med. J. 47: 1179-1181, 1973.

24. BROWN, K.H., GAFFAR, A. and ALAMGIR, S.M. Xerophthalmia, protein calorie malnutrition and infections in children. J. Peds. 95: 651 - 656, 1979.

25. MCLAREN, D.S., SHIRAJIAN, E., TCHALIAN, M., and KHOURY, G. Xerothalmia in Jordan. Am. J. Clin. Nutr. 17: 117-130, 1965.

26. WHARTON, B., HOWELLS, G., and PHILLIPS, I. Diarrhoea in Kwashiorkor. Brit. Med. J. 4: 608-611, 1968.

27. KHAN, M.U. and CURLIN, G. Shigellae dysentery: a new health hazard in Bangladesh. Bangladesh Med. J. 3: 42-48, 1974.

28. SAULSBURY, F.T., WINKLESTEIN, J.A., and YOLKEN, R.H. Chronic rotavirus infection in immunodeficiency. J. Peds. 97: 61-65, 1980.

29. BLACK, R.E., MERSON, M.H., ROWE, B., TAYLOR, P.R., RAHMAN, A.S.M.N., HUQ, MD. A., ALIM, A.R.M.A., SACK, D.A., and CURLIN, G.T. Epidemiology of enterotoxigenic Escherichia coli in rural Bangladesh. Proceedings of the 14th Joint Conference U.S.-Japan Cooperative Medical Science Program. Cholera Panel, 1978, pp. 292-301.

30. BROWN, K.H., GILMAN, R.H., KHATUN, M. and AHMED, M.G. Absorption of macronutrients from a rice-vegetable diet before and after treatment of ascariasis in children. Am. J. Clin. Nutr. 33: 1975-1982, 1980.

31. KOTCHER, E., MIRANDA, M., ESQUIVEL, R., PENA-CHAVARRIA, A., DONOHUGH, D.L., BALDIZON, C., ACOSTA, A., and APUY, J.L. Intestinal malabsorption and helminthic and protozoan infections of the small intestine. Gastroenterology, 50: 366-371, 1966.

32. BLUMENTHAL, D.S., and SCHULTZ, M.G. Effects of ascaris infection on nutritional status in children. Am. J. Trop. Med. Hyg. 25: 682-690, 1976.

33. LAYRISSE, M. and ROCHE, M. The relationship between anemia and hookworm infection. Am. J. Hyg. 79: 279-301, 1964.

34. GALVAN, R.R., and CALDERON, J.M. Deaths among children with third degree malnutrition. Am. J. Clin. Nutr. 16: 351-355, 1965.

35. CAMPBELL, J.A.H. The morbid anatomy of infantile malnutrition in Cape Town. Arch. Dis. Child. 31: 310-314, 1956.

36. STIRLING, G.A. Renal pathobology in malnourished infants. Arch. Dis. Child. 37: 378-386, 1962.

37. SUSKIND, R.M. (ed). Malnutrition and the Immune Response. Raven Press, New York, 1977.

38. Symposium on impact on nutritional status of the host. Am. J. Clin. Nutr. 30: 1203-1371, 1977.

NUTRITION RESEARCH, Vol. 1, pp. 47-62, 1981
0271-5317/81/010047-16$02.00/0 Printed in the USA.
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SOME CLINICAL INDICES OF NUTRITION AND HEALTH STATUS AMONG ELDERLY KENTUCKY RESIDENTS VOLUNTEERING TO BE SURVEYED: COMPARISON BY SOCIOLOGICAL FACTORS, LIFE STYLES AND HEALTH CHARACTERISTICS[1,2]

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ABSTRACT

From a three-county area in central Kentucky, 148 volunteers at least 60 yrs of age were studied to identify nutritional problems in relation to selected socioeconomic factors, life style and health conditions. Anthropometric measurements were made, and one fasting blood specimen from each participant was analyzed. The extent of osteoporosis of the 3-2 phalanx was determined by a densitometric scan of an x-ray of the right hand positioned beside a standard density aluminum stepwedge. Values for mean systolic blood pressure, serum total protein and bone density of blacks were greater($p < 0.05$) than those for whites. The mean levels of hematocrit, hemoglobin and bone density dropped significantly as age increased from the 60's to the 80's. Higher prevalence of hypertension and low serum ascorbic acid was observed among those living alone than among those living with a family. Higher incidences of obesity were associated with higher income group and those living with family compared to their counterparts. The prevalence of low serum ascorbic acid was observed among smokers. Persons with a history of cardiovascular diseases had significantly higher mean values of systolic blood pressure, serum triglycerides and pre-β-lipoproteins than did those without such a history. The mean bone density and serum ascorbic acid values of denture wearers were lower compared to those having their own teeth. It is suggested that agencies and individuals dealing with the nutritional well-being of the elderly carefully look into not only their socioeconomic conditions but look also into life styles of individuals being dealt with in planning, implementing and/or evaluating an action program.
KEY WORDS: nutrition survey, elderly, nutritional status

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1. Supported by Grants 616-15-126 and KY.X1979000006, Science and Education Administration, U. S. Department of Agriculture.
2. A portion of this report was presented at a meeting of the Federation of American Societies for Experimental Biology in Atlantic City, New Jersey, April, 1978. Abstract #791.

INTRODUCTION

An increasing proportion of the population in the United States consists of older individuals(1). The significance of nutrition for long life or slowing of the aging process is of great public and medical interest. In recent years, nutritional status studies on the overall population, including the elderly group, have been conducted at the national level(2, 3). However, because findings from these national surveys have not become available on a geographical basis, they have only limited usefulness. In order for these data collected by nationwide surveys to be used by existing programs to meet the needs of a community, more specific and nondiffused data are needed. Hence, this study was undertaken to quantitate nutritionally related health problems and to identify the factors associated with these problems among the elderly in central Kentucky.

MATERIALS AND METHODS

Selection and description of sample

One hundred and fifty noninstitutionalized elderly persons, between the age of 60 and 89 years residing in Franklin, Scott, or Shelby County of central Kentucky were recruited through ads in local newspapers, and on radio stations and through the county senior citizen center. Of these, 148 subjects completed the study. According to the 1970 census(1), this is equivalent to 1.22% of the population in this three-county area for this age group. However, the sample does not reflect the population in proportion to sex and race. Thus a higher proportion of females than males, and of blacks than whites participated in comparison to the population. There were 26 males of mean age 69.4 ± 5.9 years and 122 females of mean age 70.8 ± 6.6 years; there were 82 white and 66 black participants.

Data and sample collection

The survey, which was conducted by interviewers, included collection of socioeconomic data concerning family composition, age, sex, race, educational level and family income, and 28-day food intake data by record or 24-hr recall(4). On the 28th day, the subjects were brought to Doctor's Place Laboratory in Frankfort to collect fasting blood and urine specimens, and to the Kentucky State University campus to measure blood pressure, anthropometry including height, weight and triceps skinfold thickness(5), and to collect information concerning the medical history of each subject. Height was measured without shoes, and body weight was measured in light clothes, and the weight was corrected by subtracting the estimated weight of the clothes. The subjects were then taken to the radiology department, King's Daughters Memorial Hospital in Frankfort, Kentucky where an x-ray of the right hand positioned beside a standard aluminum stepwedge was taken. The blood and urine specimens collected at the clinic were transported to the Nutrition Laboratory at Kentucky State University within half an hour after the collection was made. All data were collected between the beginning of May and the end of July, 1977.

Laboratory and data analysis

The biochemical tests performed on whole blood included hemoglobin and hematocrit. Tests performed on serum included: iron, vitamin C, total protein, albumin, cholesterol, triglyceride, and lipoproteins. Urine

specimens were tested qualitatively for glucose and protein. The hemoglobin (6), serum iron(7), protein(8), albumin(9) and cholesterol(10) were determined colorimetrically by using the Clinicard 368 analyzer. The serum triglyceride was determined by Hantzach reaction(11) and serum lipoproteins were separated electrophoretically on agar-agarose films. Beta-lipoprotein and pre-beta-lipoprotein concentrations were calculated using β-lipoprotein cholesterol values in combination with the percentages of β- and pre-beta-lipoprotein determined by densitometric readings of the electrophoretic films(12,13). The remaining analytical procedures were adopted from the ICNND Manual(14). The bone density was measured by densitometric readings of the x-ray films of 3-2 phalanx in relation to that of the standard aluminum stepwedge(15,16). The body mass index, the ratio of weight/height squared, was calculated as it has been reported as the best measure of relative body weight(17,18).

Analysis of variance in combination with t-tests, and Chi-square tests were performed on the biochemical and anthropometric data to detect any significant differences due to sociological factors including race, sex, age, educational level and family income, life style such as smoking or nonsmoking and living alone or with family, and selected health characteristics such as existence of a history of heart ailments,obesity and having their own teeth or not. Correlation coefficients between selected biochemical and anthropometric data were also computed to detect whether any systematic relationships exist between variables.

This report is confined to the biochemical and clinical findings. Dietary intake data on these subjects will be reported separately.

RESULTS

Comparison by sex and race

Table 1 shows the characteristics of subjects by sex and race. Subjects ranged from 60 to 89 years of age with means of 68.4 and 70.1 years for white and black males, and 70.7 and 71.0 years for white and black females, respectively. Age and educational level did not differ significantly by race or sex. However, the mean body weight of black females was significantly($p < 0.05$) greater than that of white females. The mean height of white males was 172 cm, which was higher than 168 cm for black males($p < 0.05$); however, no difference due to race was observed in mean heights for females. The mean systolic blood pressure of 151 mmHg for black males was significantly($p < 0.005$) greater than 130 mmHg for white males, but diastolic pressures did not differ significantly. In the case of the females, both systolic and diastolic pressures of 152 and 84 mmHg, respectively, for blacks were higher than the corresponding values of 139 and 81 mmHg for whites. Unlike males, the mean bone density of black females exceeded that of whites($p < 0.005$). Of the males, 30% of the white subjects and 69% of the black were smokers, and of the females, the corresponding figures were 19% and 22%, respectively. The table also reveals the percentage of participants taking vitamin or iron supplements. Vitamin supplementations were practiced at a higher rate among white females than among black females.

The body mass index(W/H^2) was correlated to the age, triceps folds, and blood pressure of the elderly; these results are indicated in Table 2. There was a negative correlation between age and the body mass index, which was significant($p < 0.05$) for females. The body mass index was significantly correlated to the triceps, as expected. The body mass index was also positively correlated to the systolic and diastolic blood pressure among females, but did not reach a significant level among males.

TABLE 1
Characteristics of Subjects by Race and Sex

Variable	Male		p	Female		p
	White (n=10)	Black (n=16)		White (n=72)	Black (n=50)	
	Mean ± s.d.			Mean ± s.d.		
Age(yr)	68.4 ± 5.2	70.1 ± 6.4	N.S.	70.7 ± 6.5	71.0 ± 6.9	N.S.
Level of education(yr)	9.1 ± 5.0	10.2 ± 3.6	N.S.	10.5 ± 3.5	9.5 ± 3.5	N.S.
Body wt.(kg)	82.6 ± 11.7	79.7 ± 16.4	N.S.	67.5 ± 12.9	73.2 ± 14.2	0.05
Height(cm)	172 ± 4	168 ± 5	0.05	157 ± 4	156 ± 6	N.S.
Body mass index(W/H^2)	27.9 ± 4.6	28.2 ± 4.9	N.S.	27.4 ± 5.1	30.1 ± 6.0	0.05
Triceps skinfolds(mm)	18 ± 14	17 ± 15	N.S.	27 ± 9	30 ± 12	N.S.
Syst. bld. press.(mmHg)	130 ± 12	151 ± 18	0.005	139 ± 19	152 ± 21	0.005
Diast. bld. press.(mmHg)	80 ± 3	85 ± 8	N.S.	81 ± 7	84 ± 11	0.05
Bone density coefficient(mm)	5.45 ± 1.40	5.70 ± 1.90	N.S.	3.75 ± 1.55	4.90 ± 1.60	0.005
Smokers(%)	30	69	N.S.	19	22	N.S.
Use of vitamin supplements(%)	30	38	N.S.	40	24	0.05
Use of iron supplements(%)	0	6	N.S.	12	10	N.S.

N.S.; not significant

TABLE 2
Correlation Coefficients between Body Mass Index(W/H^2), and Age and Blood Pressure by Sex

Variables	Male (n=26)	p	Female (n=122)	p
W/H^2; Age	-0.3706	N.S.	-0.2223	0.05
W/H^2; Triceps	0.6534	0.01	0.7871	0.01
W/H^2; Systolic Blood Pressure	0.3606	N.S.	0.2961	0.05
W/H^2; Diastolic Blood Pressure	0.4006	N.S.	0.3877	0.01

The distribution of annual family income is shown graphically in Figure 1. A higher percentage of blacks than whites had an annual income of less than $2,400. More than half of the elderly, regardless of race, had an annual income below $5,000(64% of blacks and 61% of whites).

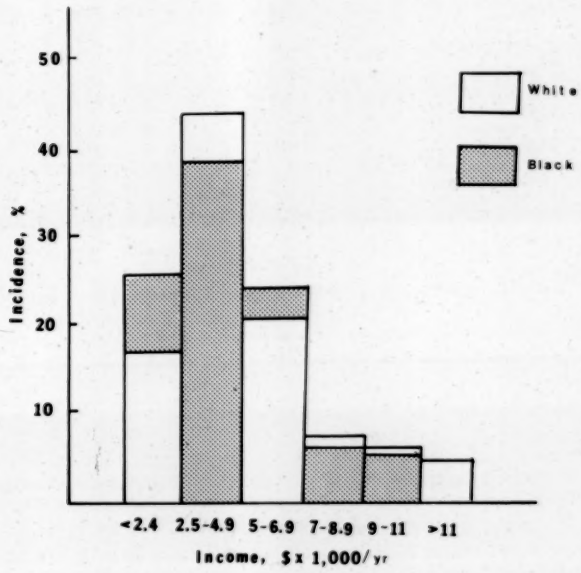

FIG. 1
Income Distribution by Race

Table 3 shows a summary of biochemical findings classified by sex and race.

TABLE 3
Summary of Hematological and Biochemical Findings by Sex and Race

Characteristic	Male		Female		p	
	White (n=10)	Black (n=16)	White (n=72)	Black (n=50)		
	Mean ± s.d.				Difference due to	
Hematocrit(%)	46 ±3	43 ±7	42 ±3	41 ±3	race sex	0.025 0.01
Hb(g/dl)	16.4 ±2.5	15.2 ±3.0	15.0 ±1.5	14.2 ±1.1	race sex	0.025 0.01
Serum level(/dl)						
iron(μg)	93 ±38	86 ±36	96 ±27	84 ±24	race sex	0.025 N.S.
vitamin C(mg)	0.75 ±0.51	0.43 ±0.34	0.89 ±0.41	0.71 ±0.41	race sex	0.05 0.01
glucose(mg)	97 ±12	106 ±35	101 ±47	109 ±34	race sex	N.S. N.S.
protein(g)	7.35 ±0.28	7.63 ±0.37	7.12 ±0.53	7.54 ±0.51	race sex	0.005 0.05
albumin(g)	3.93 ±0.26	4.01 ±0.34	4.01 ±0.34	3.98 ±0.30	race sex	N.S. N.S.
cholesterol(mg)	216 ±40	211 ±40	236 ±36	228 ±56	race sex	N.S. 0.05
triglyceride (mg)	152 ±61	104 ±69	131 ±62	116 ±69	race sex	0.05 N.S.
β-lipoprotein (mg)	251 ±61	225 ±46	247 ±49	235 ±60	race sex	N.S. N.S.
pre-β-lipo-protein(mg)	134 ±67	95 ±46	126 ±54	108 ±53	race sex	0.05 N.S.

Mean hematocrit and hemoglobin values for whites were greater than for blacks ($p < 0.025$); and for males were greater than those for females($p < 0.01$), as expected. The mean serum iron for black females was significantly lower ($p < 0.025$) than for white females, but the difference due to race among males was not significant. The serum vitamin C levels were higher($p < 0.05$) among whites and among females($p < 0.01$) than their counterparts. The serum glucose level was not significantly influenced by race or sex. There were significant differences in serum proteins due to race($p < 0.005$) and sex($p < 0.05$); thus blacks had higher values than whites, and males higher than females. However, no significant difference in serum albumin due to race or sex was observed. Mean serum cholesterol did not differ because of race, but females had significantly($p < 0.05$) higher values than males. The mean serum triglyceride values of all whites(134 mg/100 ml) was higher($p < 0.05$) than that of all blacks(113 mg/100 ml). No significant differences in serum β-lipoproteins due to race or sex were observed; however, pre-β-lipoproteins of whites were higher($p < 0.05$) than blacks.

Comparison by age group

Anthropometric and biochemical data were grouped by age of participants in decades, and tabulated in Table 4. The body weight, arm circumference, and triceps skinfolds decreased significantly($p < 0.05$) as age increased from the 60's to the 70's or to the 80's, but no difference was observed in height or blood pressure. The hematocrit and hemoglobin levels decreased significantly($p < 0.05$) as age increased from the 70's to the 80's. No difference was observed in serum levels of iron, glucose, cholesterol and triglycerides due to age. The mean bone density dropped significantly($p < 0.05$) as age increased from the 60's to the 70's and 80's. Prevalent conditions based on this age grouping are shown in Table 5. X^2 test revealed that obesities were significantly less frequent($p < 0.001$) and hypertensions were more frequent among those in the 80's than the 60's or the 70's. Hypertriglyceridemia existed more frequently among those in the 60's than the older groups($p < 0.05$).

Comparison by income level

Subjects were grouped into three categories by their annual family income level; less than $2,400, $2,401 to $5,000, and $5,001 or higher. The results are shown in Table 5. Those belonging to the lowest income level had significantly lower incidences of obesity($p < 0.05$). Differences in other conditions due to the income level did not reach a significant level.

Living alone vs. living with family

The subjects were arranged into two groups: those living alone and those living with their family. The results are also shown in Table 5. Incidences of obesity were higher among males who lived with family($p < 0.05$), and of hypertension, hyperglycemia and low serum vitamin C were higher among males who lived alone($p < 0.05$). Less prominent patterns in health characteristics between the two groups were observed among the elderly females. However, the prevalence of hyperglycemia was higher among those females who lived with a family than among those who lived alone($p < 0.05$).

TABLE 4
Anthropometric, Biochemical, Hematological, and Blood Pressure Data by Age

Condition	Age Level 60's (n=75)	70's (n=56)	80's (n=17)
		Mean ± s.d.	
Age(yr)	65.4 ± 2.3	73.7 ± 2.8	83.3 ± 2.7
Body wt.(kg)	75.7 ± 14.4[a]	68.9 ± 13.4[b]	63.0 ± 12.0[b]
Height(cm)	160 ± 7[a]	158 ± 7[a]	156 ± 6[a]
Arm circumference(mm)	320 ± 45[a]	295 ± 37[b]	269 ± 32[c]
Triceps skinfolds(mm)	29 ± 12[a]	25 ± 11[a,b]	21 ± 11[b]
Systolic blood pressure(mmHg)	143 ± 20[a]	144 ± 19[a]	152 ± 25[a]
Diastolic blood pressure(mmHg)	83 ± 8[a]	82 ± 9[a]	80 ± 10[a]
Hematocrit(%)	42 ± 4[a]	42 ± 3[a]	40 ± 4[b]
Hemoglobin(g/dl)	15.0 ± 1.9[a]	14.9 ± 1.7[a]	14.0 ± 1.5[b]
Serum iron(μg/dl)	90 ± 28[a]	93 ± 29[a]	85 ± 26[a]
Serum glucose(mg/dl)	108 ± 40[a]	99 ± 26[a]	103 ± 51[a]
Serum cholesterol(mg/dl)	224 ± 42[a]	231 ± 48[a]	245 ± 50[a]
Serum triglyceride(mg/dl)	125 ± 60[a]	126 ± 78[a]	115 ± 40[a]
Bone density coefficient(mm)	4.95 ± 1.70[a]	4.05 ± 1.75[b]	3.60 ± 1.40[b]

Means not sharing a common letter superscript are significantly different($p < 0.05$).

Smoker vs nonsmoker

The characteristics of the elderly persons were compared on the basis of smoking and nonsmoking habits. The results are summarized in Table 6. There were 39 smokers and 109 nonsmokers. There was no significant difference between the two groups in mean values for body weight, triceps, blood pressures, bone density, serum cholesterol and triglycerides. However, smokers had lower mean values in serum vitamin C($p < 0.05$) and glucose($p < 0.05$), while the nonsmokers had a lower mean value in hematocrit($p < 0.05$) compared to their counterparts.

TABLE 5
Prevalent Health Conditions by Age, Income and Selected Life Style(%)

	Age Level, yr.			Income Level			Living Alone		Living with Family	
	60's(75)	70's(56)	80's(17)	I(31)	II(62)	III(57)	Male(9)	Female(69)	Male(17)	Female(53)
Obesity	53[a]	43[a]	12[b]	26[a]	45[b]	50[b]	0[a]	45[b]	35[b]	53[b]
Hypertension	25[a]	29[a]	53[b]	42[a]	27[a]	24[a]	44[a]	32[a,b]	24[a,b]	19[b]
Low HCT	16[a]	23[a]	29[a]	10[a]	8[a]	15[a]	33[a]	12[a,b]	47[a]	6[b]
Hyperglycemia	16[a]	5[a]	12[a]	10[a]	10[a]	13[a]	33[a]	7[b]	0[b,c]	19[a,c]
Hypercholesterolemia	23[a]	25[a]	35[a]	32[a]	18[a]	25[a]	11[a]	25[a]	18[a]	25[a]
Hypertriglyceridemia	17[a]	9[a,b]	0[b]	10[a]	9[a]	19[a]	0[a]	7[a]	29[b]	15[a,b]
Low serum vit. C	4[a]	9[a]	0[a]	7[a]	7[a]	2[a]	33[a]	4[b]	0[b]	0[b]
Osteoporosis	75[a]	78[a]	82[a]	81[a]	79[a]	69[a]	67[a]	88[a]	47[a]	75[a]

I, II, III designate the annual family income level of less than $2,400, $2,401 to $5,000, and higher than $5,000, respectively. Values not sharing a common letter superscript are significantly different($p < 0.05$).

TABLE 6
Characteristics of smokers and nonsmokers

	Smoker*(39)	Nonsmoker(109)	p
	Mean ± s.d.		
Body wt.(kg)	70.2 ± 14.0	72.2 ± 14.5	N.S
Height(cm)	161.0 ± 7.0	158.0 ± 6.75	N.S.
Triceps skinfolds(mm)	24 ± 13	27 ± 11	N.S.
Systolic blood pressure(mmHg)	140 ± 18	146 ± 21	N.S.
Diastolic blood pressure(mmHg)	82 ± 7	82 ± 9	N.S.
Bone density(mm)	4.5 ± 1.8	4.5 ± 1.7	N.S.
HCT(%)	43 ± 5	41 ± 3	0.05
Serum iron(μg/dl)	93 ± 35	90 ± 25	N.S.
Serum vitamin C(mg/dl)	0.65 ± 0.37	0.82 ± 0.45	0.05
Serum glucose(mg/dl)	96 ± 16	107 ± 41	0.05
Serum cholesterol(mg/dl)	221 ± 52	233 ± 43	N.S.
Serum triglyceride	113 ± 56	130 ± 69	N.S.

*Frequency of smoking ranged from 5 to 40 cigarettes per day with a mean of 16 ± 9, and the duration of smoking ranged from 7 to 74 yrs with a mean of 36.3 ± 16.2 yrs.

Presence or absence of a history of cardiovascular disease

The participants were divided into two groups, with and without a history of cardiovascular diseases including heart failure or angina pectoris, associated with atrial enlargement, ventricular hypertrophy, myocardial infarction, or atherosclerosis. There were 44 persons who had a history of such conditions. The findings are tabulated in Table 7. Persons with a history of cardiovascular disease had significantly higher values in systolic blood pressure, serum triglycerides and pre-β-lipoproteins.

TABLE 7
Comparison of Persons With and Without a History of Cardiovascular Disease

Condition	Person with cardiovascular disease (n=44) Mean ± s.d.	Absence of cardiovascular disease (n=106) Mean ± s.d.	p
Age(yr)	71.8 ± 6.3	70.0 ± 6.5	N.S.
Triceps skinfolds(mm)	26 ± 13	26 ± 11	N.S.
Blood pressure(mmHg)			
systolic	151 ± 23	141 ± 19	0.01
diastolic	83 ± 10	82 ± 8	N.S.
Serum lipids(mg/100 ml)			
cholesterol	227 ± 59	231 ± 39	N.S.
triglycerides	143 ± 74	107 ± 60	0.05
β-lipoproteins	248 ± 59	238 ± 51	N.S.
pre-β-lipoproteins	130 ± 55	111 ± 52	0.05

Denture wearers vs persons with own teeth

The results from grouping of the elderly persons into one group having their own teeth and the other having complete dentures are tabulated in Table 8. There was no significant difference between the two groups in mean values for triceps skinfolds, blood pressure, hematocrits, hemoglobin, and serum levels of iron, serum cholesterol, and β- and pre-β-lipoprotein. However, mean serum triglycerides($p < 0.05$), bone density($p < 0.05$) and serum vitamin C($p < 0.01$) of those having their own teeth were higher than those of full denture wearers.

TABLE 8
Comparison Between Elderly Having Teeth of
Their Own and Complete Dentures

Condition	Elderly with own teeth (n=52) Mean ± s.d.	Elderly with dentures only (n=96) Mean ± s.d.	p
Triceps skinfolds(mm)	25 ± 11	27 ± 12	N.S.
Systolic blood pressure(mmHg)	140 ± 19	147 ± 21	N.S.
Diastolic blood pressure(mmHg)	83 ± 7	82 ± 9	N.S.
Hematocrit(%)	42 ± 4	42 ± 4	N.S.
Hemoglobin(g/dl)	14.8 ± 2.1	14.9 ± 1.6	N.S.
Serum iron(μg/dl)	96 ± 32	88 ± 26	N.S.
Serum vitamin C(mg/dl)	0.91 ± 0.44	0.70 ± 0.41	0.01
Serum glucose(mg/dl)	96 ± 19	108 ± 43	0.05
Serum cholesterol(mg/dl)	232 ± 36	228 ± 50	N.S.
Serum triglyceride(mg/dl)	139 ± 76	116 ± 58	0.05
Serum β-lipoprotein(mg/dl)	249 ± 55	237 ± 53	N.S.
Serum pre-β-lipoprotein(mg/dl)	128 ± 61	111 ± 48	N.S.
Bone Density coefficient(mm)	4.85 ± 1.50	4.25 ± 1.80	0.05

DISCUSSION

The mean heights of elderly persons of both races and sexes in the present study are in agreement with nationwide statistics(19). However, the mean body weights of males of both races and of black females in the present study are higher than the national average for this age group. Higher incidences of obesity were associated with females, among younger groups than older ones, among smokers than nonsmokers, and among those living with family than being alone. The mean systolic blood pressure of blacks of both sexes was comparable to corresponding data collected by the HANES study(20). However, mean systolic blood pressure of whites of both sexes, and mean diastolic blood pressure of both races and of sexes of the present study were lower than the national mean. It is possible that less tense and more relaxing environmental conditions in central Kentucky compared to highly urbanized areas might have

contributed to those lower values in blood pressure. Prevalences of hypertension increased with age level, were higher among blacks than whites, those living alone than with family, and those having a history of heart ailments than their counterparts.

Higher mean values in bone density of males than females, and of blacks than whites are in good agreement with findings by others(15, 21). Higher incidences of osteoporosis were associated with older groups than younger groups of the elderly, with females than males, among whites than blacks, and those living alone than those with family. Among the health conditions studied in the present study, osteoporosis was most prevalent. The magnitude of this disorder, which reached as high as 92% among white females, should not be overlooked. A recent report(16) has shown a close statistical correlation between the incidence of subnormal bone density, fracture risk, and lower calcium consumption of women over 45 years of age in the United States. However, whether osteoporosis among the elderly should be regarded as a bone disease of nutritional origin is yet to be clarified. Studies of sufficient duration to provide adequate evaluation of a nutritional approach to the problem are needed.

The mean values of hemoglobin and hematocrit of the present study were higher, and the mean serum iron levels were lower, than data reported in the nationwide survey(22). However, the incidences of low or deficient values reported for the elderly are still in agreement in both studies. The lower mean values in hemoglobin and hematocrit for blacks compared to those for whites have also been well documented(3, 22). Whether or not the evaluation of hemoglobin and hematocrit values among blacks requires a different set of standards than among whites remains to be clarified with controlled experiments. The greater frequency of low hematocrit values among males compared to females, in spite of the higher mean hematocrit values for males, is due to use of separate standards for males and females.

The mean serum protein levels of the present study were similar to those reported by the Ten-State(22) and by the HANES study(3). The mean albumin values of the present study were lower than those reported in the above studies; however, they were in accord with values reported by others(23). The incidence of low albumin level in serum was slightly higher for participants of the present study compared to the national rate(3).

The mean serum glucose levels are in agreement with data reported for people 56 to 85 years of age in Macon County, Alabama(24). In the present study, the incidences of hyperglycemia and persons with diabetes mellitus were higher among people in the 60's than the older groups, among blacks than whites, among denture wearers than those having their own teeth, and among males living alone compared to their counterparts.

The mean serum cholesterol levels are slightly lower than those reported on the U.S. population(25). Higher serum cholesterol values were associated with older age, smokers and females when these were compared to their counterparts. Mean serum triglyceride levels of the present participants were similar to those in Alabama for the same age group(24). Higher incidences of hypertriglyceridemia were associated with whites, denture wearers, those living with family, and those having a history of heart ailments. It is interesting to observe that none of the persons above 80 years of age had hypertriglyceridemia, and the rates were higher among those in the 60's than those in the 70's. Unlike hypercholesterolemia, triglyceridemia was not more frequent among smokers than nonsmokers in the present study.

Low serum vitamin C levels were associated with those living alone, denture wearers and males, particularly smoking males. It is conceivable that

males and those living alone do not prepare a sufficient variety of foods, and that consumption of fruits and vegetables is limited among the males and denture wearers. Lower plasma levels of vitamin C among smokers compared to nonsmokers have been well documented by others(25, 26, 27).

This study has revealed that nutritional problems among the elderly are compounded not only by low family income and age of individual, but by their health conditions and life styles such as whether person lived alone or with family, whether the person smoked or not, and whether the person had a history of heart ailments or not. These findings should be useful to individuals involved with the elderly and their well-being.

ACKNOWLEDGMENTS

The senior author acknowledges the cooperation of the elderly participants. Technical assistance of Beth Clark, Terry Kelly, Peggy Rexroat, Devora Street and Janice Wade is recognized.

REFERENCES

1. U. S. Bureau of Census. 1970 Census of Population PC(V2)-19 Kentucky. Advance Report: General Population Characteristics. U. S. Government Printing Office, Washington, D. C. 1971.

2. U. S. Department of Health, Education and Welfare. Ten-State Nutrition Survey 1968-1970. I. Historical Development and II. Demographic Data. DHEW Publication No.(HSM)72-8130. Center for Disease Control, Atlanta, Ga., 1972.

3. U. S. Department of Health, Education and Welfare. Preliminary Findings of the First Health and Nutrition Examination Survey, United States, 1971-1972: Dietary Intake and Biochemical Findings. DHEW Publication No.(HRA)76-1219-1, Health Resources Administration, Rockville, Md., 1974.

4. LUND, L. A. and BURK, M. C. A Multidisciplinary Analysis of Children's Food Consumption Behavior. Technical Bulletin No. 265. Agricultural Experiment Station, University of Minnesota, Minneapolis, 1969.

5. U. S. Department of Health, Education and Welfare. Anthropometric and Clinical Findings. Preliminary Findings of the first Health and Nutrition Examination Survey. U. S., 1971-72. DHEW Publication No.(HRA)75-1229. National Center for Health Statistics, Rockville, Md., 1975.

6. The Clinicard 32202 Hemoglobin Chemistry. Harleco, A Division of American Hospital Supply Corporation, Gibbstown, N. J., 1974.

7. The Clinicard 32206 Iron Chemistry. Harleco, A Division of American Hospital Supply Corporation, Gibbstown, N. J., 1975.

8. The Clinicard 32101 Total Protein Chemistry. Harleco, A Division of American Hospital Supply Corporation, Gibbstown, N. J., 1975.

9. The Clinicard 32107 Albumin Chemistry. Harleco, A Division of American Hospital Supply Corporation, Gibbstown, N. J., 1974.

10. The Clinicard 32104 Cholesterol Chemistry. Harleco, A Division of American Hospital Supply Corporation, Gibbstown, N. J., 1975.

11. FOSTER, L. B. and DUNN, R. Y. Stable reagents for determination of serum triglycerides by a colormetric Hantzach condensation method. Clinical Chemistry, 19: 338-340, 1973.

12. KREHL, W. A. LOPEZ-S, A. and GOOD, E. I. A rapid analytical system for determining serum lipids. American Journal of Clinical Nutrition, 20: 968-977, 1967.

13. LOPEZ-S, A. VIAL, R. GREMILLION, L. and BELL, L. Automated turbidimetric determination of beta-plus-pre-beta-lipoprotein cholesterol. Clinical Chemistry, 17: 994-997, 1971.

14. Interdepartmental Committee on Nutrition for National Defense, Manual for Nutrition Surveys(2nd ed.), U. S. Government Printing Office, Washington, D. C., 1963.

15. ALBANESE, A. A. Chapter 6, Detection of Bone Loss - Physical Method. In: Bone Loss - Causes, Detection, and Therapy. Current Topics in Nutrition and Disease. Vol. 1. Alan R. Liss, Inc., New York, 1977.

16. ALBANESE, A. A. EDELSON, A. H. LORENZE, E. J. JR. WOODHULL, M. L. and WEIN, E. H. Problems of Bone health in elderly, ten year study. New York State Journal of Medicine, 75: 326-336, 1975.

17. KEYS, A. FIDANZA, F. KARVONEN, M. J. KIMURA, N. and TAYLOR, H. L. Indices of relative weight and obesity. Journal of Chronic Diseases, 25: 329-343, 1972.

18. KEYS, A. Overweight, obesity, coronary heart disease and mortality. Nutrition Review, 38: 297-307, 1980

19. U. S. Department of Health, Education and Welfare. Weight and Height of Adults 18-74 years of age: United States, 1971-74. DHEW Publication No.(PHS)79-1659. National Center for Health Statistics. Hyattsville, Md., 1979.

20. U. S. Department of Health, Education and Welfare. Blood Pressure Levels of Persons 6-74 years. United States, 1971-1974. DHEW Publication No.(HRA)78-1648. National Center for Health Statistics. Hyattsville, Md., 1977.

21. GSELL, O. R. Longitudinal gerontological research over 10 years(Basel Studies, 1955-1965). Gerontologica Clinica, 9: 67-80, 1967.

22. U. S. Department of Health, Education and Welfare. Ten-State Nutrition Survey, 1968-1970. IV. Biochemical. DHEW Publication No. (HSM)72-8132. Center for Disease Control. Atlanta, Ga., 1972.

23. STIEDMAN, M. JANSEN, C. and HARRILL, I. Nutritional status of elderly men and women. Journal of the American Dietetic Association, 73: 132-139, 1978.

24. PROTHRO, J. MICKLES, M. and TOLBERT, B. Nutritional status of a population sample in Macon County, Alabama. American Journal of Clinical Nutrition, 29: 94-104, 1976.

25. U. S. Department of Health, Education and Welfare. Total Serum Cholesterol Level of Adults 18-74 years of age, United States, 1971-74. Advance data from Vital and Health Statistics of the National Center for Health Statistics. No. 7, Health Resources Administration. Rockville, Md., 1977.

26. BROOK, M. and GRIMSHAW, J. J. Vitamin C concentration of plasma and leucocytes as related to smoking habits, age, and sex of humans. American Journal of Clinical Nutrition, 21: 1254-1258, 1968.

27. PELLETIER, O. Vitamin C status of cigarette smokers and non-smokers. American Journal of Clinical Nutrition, 23: 520-524, 1970.

NUTRITION RESEARCH, Vol. 1, pp. 63-71, 1981
0271-5317/81/010063-09$02.00/0 Printed in the USA.
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"MODERN" AND "TRADITIONAL" FOOD USE IN WEST FINLAND: AN EXAMPLE OF QUANTITATIVE PATTERN ANALYSIS

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ABSTRACT

This paper presents a method for pattern analysis of food use behavior. Data from a small city in the western part of Finland are presented to illustrate the dimensions of "traditional food use" and "modern food use" in a community experiencing rapid modernization and processing. The two scales of "traditional food use" and "modern food use" were derived through a process that involved ethnographic research and the application of factor analysis and scale construction to food intake data. The scales proved to be statistically independent, suggesting that changes in food use do not follow a simple linear pattern.

KEY WORDS: dietary change, pattern analysis of food use

INTRODUCTION

The description of food use patterns in terms that are both ethnographically-sensitive and quantitative (i.e. amenable to hypothesis-testing) presents a considerable challenge for nutritional anthropology. The mode of pattern analysis utilized here to contrast "modern" and "traditional" food use in West Finland represents one technique for quantifying cultural dimensions of diet. While scale construction is used here to characterize patterns that are related to social change, the technique is applicable to a variety of food- and nutrition-related concepts.

Food Use in West Finland in the Twentieth Century

Until the decade of the 1960s, food consumption in rural, western Finland reflected a centuries-old pattern of dependence on dairy products, grains and potatoes, the latter introduced into Finland in the 1760s. Even today the Finns rank among the leading milk-consuming nations of the world. The traditional diet was characterized by high-protein, high-calorie intake, with few fruits and leafy vegetables.

Before the 20th century Finland remained relatively isolated from international trade, especially in foodstuffs, and diet was based mainly on local resources. Although the southern and southwestern parts of Finland had many large landholdings, owned principally by the Swedish-speaking aristocracy, the central and western provinces were dominated by small- and middle-sized freeholders with their numerous crofters (tenant farmers). Families depended on small-scale subsistence dairy farming, i.e. one or two cows, a potato patch and small fields of rye and barley. Purchased foods in this area were confined mainly to sugar, tea, salt, coffee, rice (for desserts) and a few spices.

Fish were important mainly in coastal areas and were carried inland by peddlers, particularly in connection with special seasonal catches and the ubiquitous dried cod (Swed. *lutefisk*; Finn. *lipeakalaa*) of Christmas dinner. Meat from wild game was of diminished importance in the 20th century, and the occasional meat from pigs and young bulls slaughtered in the Fall was used as sausage and preserved by salting and pickling.

In the first half of this century dietary change proceeded slowly as economic conditions did not improve significantly, although Finland was gradually becoming part of the European economic, industrialized community. Until World War II the Finnish economy was an underdeveloped backwater in the European socioeconomic system. The war dealt hard blows to the economy and social order as the Finns ended up on the losing side. Immediately after World War II, the Finnish economy was burdened (and stimulated) by heavy "war reparations" demanded by the Soviets. It was a time of austerity in food as with other products. Those of us who were travelers in Finland in the mid-50s noted that there were occasionally apples and oranges, sometimes even bananas, but these were relatively unusual, specialty items.

The 1960s brought rapid growth of economic prosperity and a large growth of imports in a wide variety of consumer goods, including foodstuffs. Economic ties with Eastern Block countries resulted in imports of apples, pears and other fruit, in season. Citrus fruits became common throughout the years, imported from Mediterranean countries. Domestic production of tomatoes and cucumbers -- mainstays of summertime salads -- increased rapidly. The advent of plastic made it possible to construct inexpensive greenhouses with heating systems, further increasing the availability of tomatoes, cucumbers and other previously scarce vegetables.

The growth of food-processing industries came quite slowly to Finland and did not have large effects on diet until the 1960s and 1970s. Of the new commercial products, perhaps the most widely advertised and most controversial is *makkara* (sausage). As mentioned, sausage was part of the traditional diet, but only as an occasional specialty item. Sausage-making is a slow and tedious process, and traditionally only wealthier homes had the available meats and the personnel to maintain extensive supplies (1). Commercially produced sausage was already common in the 1950s and grew in popularity, in part because of the continued inflation of meat prices.

The major food chains in Finland include both private entrepreneurs and large-scale cooperatives. The nation-wide producers' cooperatives are, in some respects, the most expansive and innovative commercial food distributors, and sausages have become a major expansion area in their retail stores. At the same time, lifestyle changes have played a part in the picture of dietary change. Camping, cook-outs and picnics have become important summertime activities for a large sector of the population. Special varieties of sausages have been developed for these various forms of recreation, in addition to the widespread use of *sauna-sausage*, taken with the weekly or twice-weekly steambath. The rapid spread of electric grills and barbecues provides additional opportunities for pattern development. The "sausage culture" is perhaps the most negatively viewed aspect of "dietary modernization," particularly by nutritionists in Finland.

Beginning in the 1960s and intensifying in the 1970s, nutrition education became important in Finland. Concern about high mortality rates from heart disease and other cardiovascular illness led to campaigns against high intakes of fat-rich dairy products and sausages, also while emphasizing the value of citrus fruits, leafy vegetables and other vitamin-rich foods. Nutrition education and health campaigns in Finland are generally well organized.

Their messages are spread through an efficient school system, national television and radio networks, and local homemakers' organizations. The nationalized health care system also participates in dietary campaigns, and newspapers and periodicals carry frequent articles about healthy foods and suggestions for dietary change.

Concepts of Modern and Traditional Food Use

Studies of changing food consumption patterns generally seek to identify major patterns and regularities of diet that affect nutritional outcomes. In most parts of the world the list of available foods is changing rapidly as the spread of commercial food distribution systems and involvement in a cash economy penetrates ever farther into formerly semi-isolated regions. The spread of commercially-marketed foods is often highly correlated with losses of locally produced foods because of reduction of wild products (2), economic deterioration and/or conversion to specialized cash crops (3,4). In more affluent sectors, the spread of new food processing equipment is also transforming food use patterns. Few, indeed, are the "pristine" communities with stable, unchanging dietary patterns.

Nutritional anthropologists and community nutritionists have used a number of different approaches in defining the stable and the newly emerging patterns of food use. Often research attention is focused on a few main staple foods; in some cases the dietary patterns are not examined at all because food intake data are converted directly into nutrient estimates. Some researchers have utilized constructs such as "core foods" and "secondary core" (5,6). Jelliffe (7) and others have used the concept of cultural "super foods" descriptively, but this usually applies only to one main element rather than to an entire food use style. "Food acculturation" and "modernization" have been explored by some nutritional anthropologists but the descriptions have usually been in qualitative terms, which do not permit statistical hypothesis-testing and do not attempt to quantify the range of variation in utilization of the patterns.

Although it is commonly assumed that "traditional" and "modern" are two ends of a single dimension or continuum, there is increasing evidence that change is more complex. In food use, as in other aspects of lifestyle, an individual does not necessarily have to give up old patterns when new usages are adopted. When people have the economic means to explore "modern" foods they can, theoretically, simply add variety to long-standing dietary selection modes. The recent "cultural revivalism," in Finland and elsewhere, might actually bring about some intensification of traditional food practices, even as people add on the use of fruits, vegetables and other elements of "modernism." Other researchers have noted the partial independence of "traditional" and "modern" features in health care (8), food production (9), Indian cultural characteristics (10) and ethnic identity (11).

We therefore hypothesized that "traditional food use" and "modern food use" would produce *separate* patterns in our research population in West Finland and that these separate dimensions would be associated statistically with different constellations of background variables. The "traditional food use" pattern, we hypothesized, should include such elements as a strong reliance on milk products, grain products in bread and porridge, and adherence to the coffee-and-coffee bread ritual that has been well-established since the turn of the century. The "modern food use" pattern, on the other hand, was expected to consist of integrated use of citrus fruits, green vegetables, other fruits, salads, and reliance on sausages.

It is important to mention that these food use patterns are required to

have *both* conceptual, "face validity" *and* statistical coherence. The first of these is simply based on the logical fit with background ethnographic information. The second depends on the statistical processes of scale construction, to be described below.

METHODS AND RESULTS

The Research Process

Our ethnographic knowledge of Finnish food culture derives from several years of previous research experience in Finland and Finnish Lapland. These contacts with Finnish culture began in 1954-55 (one year in residence) and include extensive field work in 1958-59, 1962, 1963, 1967, and several visits in the 1970s. Seven months of residence and research in 1978 was focussed on food use and nutrition. In addition to a social and nutrition survey, we interviewed residents of West Finland at length, conferred with nutritionists and ethnographic researchers, observed numerous home-cooked meals, observed different kinds of food stores and made lists of prices of the available items.

The construction of detailed interview schedules and other instruments requires a considerable investment in descriptive, ethnographic research. Knowledge of the local food system in economic, social and cultural terms is essential for effective preparation of quantified data-gathering. The ethnographic information provides one with hypotheses about the content and organization of "traditional" and "modern" practices. Ethnographic fieldwork also provides information about what kinds of population sectors are likely to be available for interviewing, when they are likely to be unavailable and other logistical features important to the research process.

A structured interview schedule was developed from the ethnographic background materials and was pre-tested in the west Finish city of Seinäjoki. Seinäjoki was chosen as the research site because it is a rapidly growing market center population (population approximately 25,000) with a small core of long-term residents. A large majority of the current population are young, upwardly mobile working- and middle-class people who still maintain ties to their rural origins in the nearby agricultural communities.

The Research Population and Sample

In order to select a random sample of respondents from the Seinäjoki population, which consists of some 4,000-5,000 households, we divided the residential sectors into approximately 30 neighborhoods. Usually this process involved subdividing named subcommunities into smaller units on the basis of more or less natural divisions (larger streets, other natural features). We then randomly selected a series of these subcommunities, separately in the presumed low-income and middle-income neighborhoods. Interviewers then followed a randomizing process in selecting *individual* household units within a given subsector. The sampling procedure was designed to obtain approximately equal numbers from working class and middle income residential sectors.

In Seinäjoki, as in most Finnish cities, there is no distinctly poor sector, and residential areas are not sharply distinctive in terms of socioeconomic status. There are small numbers of Gypsies and Sami but they constitute only a tiny fraction of the population. At the upper end of the scale, there is a small professional-managerial "elite", but their levels of wealth and styles of life are not sharply distinct from the upper middle income people. Very few families in Seinäjoki inherited wealth. The cultural separation of Swedo-Finnish and Finnish individuals and families is important in many

coastal cities, but is not of significance in Seinäjoki, which is overwhelmingly Finnish.

In addition to the random sample of 60 households in the city, we also interviewed 48 householders randomly selected in 4 rural communities within a few kilometers of Seinäjoki. These are old agricultural communities, rather than newly-built suburban residential areas. Because access from these rural communities into Seinäjoki is quite easy by bus transportation and private automobile, this "rural fringe" population is not sharply distinct from the city population.

The sample of 108 households was thus obtained through a two-stage, randomized "cluster" process. The stratification of the sample into "middle income" and "somewhat lower income" residential sectors is not involved in the data analysis presented here. The overall (relative) homogeneity of cultural background and socio-economic status in the Seinäjoki population made the sampling process quite unproblematic.

Interviewers were four university students enrolled in the ethnology-anthropology program. They were all familiar with west Finnish culture and had previous field research experience. Their socio-economic backgrounds were in approximately the middle range of income levels of Seinäjoki, but their "graduate student" status placed them in a near-elite category in terms of educational attainment.

In all cases the interviews were carried out with women who were identified as the "female household head." In this analysis the food intake dataare in the form of a 24-hour recall. The interviews, food diaries and recalls that comprise all the components of the survey were collected over a five-month period that included both winter and early summer seasons.

Steps One in Scale Construction: Coding the Variables

For purposes of developing the two scales of "traditional food use" and "modern food use," we first coded the food items from the 24-hour recalls of the 108 respondents. These food variables were developed, in part, from standard dietary categories used by nutritional scientists (e.g. citrus fruits, milk, milk products). Other categories were based on culturally-specific and ethnographically-sensitive items. For example, coffee and "*pullaa*"(coffee bread) represent the distinctive coffee routine of Finns (12). Similarly, the category of *makkaraa* (commercially prepared sausages) is listed separately, rather than included under servings of high protein foods, because of its ethnographic importance.

For purposes of this kind of pattern analysis the number of servings is sufficiently quantitative, as contrasted with more precise intake measures. We are not concerned here with measuring nutrient intake but rather with the *frequency of appearance* of particular foods. In some instances, however, more precise information has been coded, so that those items can be expressed in terms of percentages of recommended daily servings. In a later stage of analysis, these items were converted into standard scores using the following formula: score minus the mean, divided by the standard deviation.

After coding, descriptive statistics were run on each diet variable in order to be sure that they showed sufficient variability to be utilizable for further statistical analysis. The items in Table 1 are illustrative of the distributions, as well as interesting descriptively, although they should be interpreted with caution since they are based on a single 24-hour recall.

TABLE 1
Distribution of Consumption Selected Food Items

	Number of servings						
Item	0	1	2	3	4	5	6+
Coffee	7[a]	5	14	25	18	6	33
Milk	29	30	32	17[b]			
Coffee-bread	48	27	17	16[b]			

[a] Numbers throughout refer to individuals.
[b] Coded as "3 or more."

With regard to more complex food items, such as citrus fruits, for which no single, uniform serving unit applies, consumption is reported as a percentage of the recommended daily serving. Thus, for example, among our 108 respondents, 58 consumed between 0 to 25% of recommended daily amount of citrus fruits, 38 consumed 25 to 100% and 12 consumed over 100% for the recall period.

Step Two: Factor Analysis

After creating 17 dietary variables, and coding and examining the distributions, the next step was to enter them into a factor analysis in order to identify foods that cluster together statistically. The varimax rotation, with iterations produced four factors, of which the first two were particularly clear and interpretable, with eigen values of 3.19 and 2.41 respectively. Inspection of the factor loadings reveals that the two factors represent coherent assemblages that correspond to our "modern food use" and "traditional food use" hypotheses. Together, these two factors account for 48% of the variance in the 17 dietary variables. Table 2 shows the dietary items in these two factors.

TABLE 2
"Modern" and "Traditional" Food Use Factors
(Factor loadings in parentheses)[a]

Factor I. (Modern Food Use)	Factor II. (Traditional Food Use)
Variety of vegetables (0.80)	Grains [in bread, porridge] (0.66)
Servings of vegetables (0.65)	Sugar [foods high in sugar] (0.61)
Salad servings (0.58)	Glasses of fluid milk (0.61)
Citrus fruits (0.51)	Cheese and fermented milk (0.51)
Variety of fruits (0.42)	Coffeebread servings (0.46)
Sausage servings (0.42)	Coffee [number of cups] (0.44)
High protein foods (0.38)	Butter and margarine (0.41)
	Potato servings (0.36)
	Fresh fruit (-0.34)

[a] Inclusion in a factor required a factor loading $\geq$ 0.30.

Step Three: Scale Analysis

The food items most significantly associated with the two major factors provide us with the tentative contents of the two hypothesized scales. The scales were created by summing an individual's (standardized) scores for each of the items selected for inclusion in the scale.

Two major food sources are not included in either the traditional food use or the modern food use scale. These are the basic items of potatoes and meat/fish/eggs, coded as "high protein foods." As can be seen in Table 2, high protein foods are clearly related to the modernization factor (factor loading of 0.38). Potatoes are equally clearly linked to the traditional food use factor (factor loading of 0.36). However, it is our judgement that these items are particularly sensitive to differences in economic means; at the same time in one form or another they are part of practically all Seinäjoki diets. (In future analysis we will examine the ways in which *variations* in use of potatoes and "high protein sources" correlate with other aspects of diet and lifestyle.)

The "Modern Food Use Scale", with six items (citrus, sausage, salad, variety of vegetables, servings of vegetables and variety of fruits) had a possible range of scores from 0 to 10. The traditional scale, consisting of seven items (grains, butter, sugar, fluid milk, cheese and fermented milk products, coffee and coffee bread), had a possible range of scores from 0 to 11. The scales were then tested for internal statistical coherence by means of Cronbach's alpha, using a standard SPSS computer routine (13). None of the items failed the test as the Modern Food Use Scale achieved an alpha level of .67 and the Traditional Food Use Scale reached an alpha of .65, both above the generally accepted cut-off level of .60. Table 3 shows the distribution of scores for the two scales.

TABLE 3
Distribution of Scores on Traditional and Modern Food Use Scales

	Score											
Scale	0	1	2	3	4	5	6	7	8	9	10	11
Modern[a]	7[c]	9	8	11	16	16	17	10	5	7	2	--
Traditional[b]	2	7	12	10	11	15	15	16	4	12	2	2

[a] Mean = 5.66; mode = 6.00.
[b] Mean = 9.08; mode = 7.00.
[c] Numbers throughout refer to individuals.

Finally, to test the hypothesis that traditional and modern food use patterns exist as independent dimensions of behavior, the degree of association between the two scales was examined with a Pearson correlation coefficient. As predicted, there is *no* correlation between the two scales ($r = 0.03$). Thus, the two food use patterns are independent.

DISCUSSION AND CONCLUSIONS

From the variety of foods currently eaten by people in the West Finnish area of Seinäjoki, two major food use dimensions emerged. One of these, the "traditional food use scale" reflects the constellation of traditional foods that had become the predictable, stable diet of rural as well as modern income urban Finnish families in the period before the Second World War. Dairy products and grain had been the long-term mainstays of Finnish diet over the centuries, while sugar, coffee and *pullaa* represent twentieth century refinements that had come to be "traditional" before World War II.

The "Modern Food Use Scale" represents the collection of foods that have emerged rapidly in the 1960s and 1970s -- a period of affluence and economic growth. There are two types of items in this scale: commercially prepared sausage (the Finnish answer to hamburgers and hotdogs) and a great variety of fruits and vegetables.

In the conventional wisdom of earlier studies, it would be expected that one would find an *inverse* relationship between the traditional food use pattern and the modern trends. Many theoretical works on "acculturation", "modernization" and related concepts present models of a unidimensional continuum, in which the addition of modern features generally occurs at the expense of traditional content. However, in the Finnish context modern and traditional eating patterns do not contradict each other. Traditional foods continue to represent a major element of diet, but they do not prevent the adoption of new cultural food components.

This paper presents a mode of pattern analysis for the quantitative description of cultural components of food use. The method itself is simple and is widely used in other disciplines for quantifying cultural/behavioral information. The methodology described here is not restricted to dimensions of dietary change. Other kinds of patterns, such as "degree of ethnic identity in food use" or "degree of vegetarianism", may be examined using these same techniques.

In order to facilitate testing of hypotheses that link sociocultural characteristics to diet and nutritional outcomes, we need to utilize quantitative methods of pattern analysis that have the following characteristics:

a) ethnographic sensitivity, which means that the research process has to include ethnographic and historical components,

b) the ability to capture the range of variation (of individuals, households or larger units) in the pattern,

c) the possibility for testing reliability and validity.

The method of scale construction presented here illustrates one type of quantitative pattern analysis. This descriptive analysis also provides a base from which to measure future changes in West Finnish dietary patterns related to "modern" and "traditional" food use.

ACKNOWLEDGEMENTS

We wish to acknowledge our gratitude to the *Kulttuurirahasto*/(Cultural Fund of Ostrobotnia) and the University of Connecticut Research Foundation for generous support of this project. We also are much indebted to Professors Antti Ahlstrom and Leena Räsänen at the Institute for Nutrition, University of Helsinki, and to Hilkka Uusivirta of the Hotelli-ja Ravintolamuseo (Hotel and Restaurant Museum in Helsinki) for providing us with much information about Finnish nutrition research and Finnish food culture. We also express our warm appreciation of the fine field work carried out by our assistants, university students Kristiina Eronen, Raili Kalliovaara, Jarmo Kankaanpää, Ritva Päivärinne, and Aulikki Ritari. The kind support of the Seinäjoki Home Economics Agency and the city municipal office also contributed to the success of our research.

REFERENCES

1. TALVE, I. Suommen Kansanomaisesta Roukataloudesta. Turun Yliopiston Kansatieteen Laitoksen Toimituksia. 2. Turku, Finland, 1973.

2. MESSER, E. The ecology of vegetarian diet in a modernizing Mexican community. In: Nutrition and Anthropology in Action. T.K. Kitzgerald (ed). Van Gorcum, Amsterdam, 1977, pp. 117-124.

3. NIETSCHMANN, B. Hunting and fishing among the Miskito Indians, Eastern Ecuador. Human Ecology, 1: 41-67, 1972.

4. GROSS, D. and UNDERWOOD, B. Technological change and caloric costs: sisal agriculture in northeast Brazil. Am. Anthro., 73: 725-740, 1971.

5. BENNETT, J.W. Food and culture in Southern Illinois. Am. Socio. Review, 7: 645-660, 1942.

6. JEROME, N.W. Diet and acculturation; the case of Black American immigrants. In: Nutritional Anthropology. N.W. Jerome, R.F. Kandel and G.H. Pelto (eds). Redgrave, Pleasantville, N.Y., 1980, pp. 275-326.

7. JELLIFFE, D.B. Quoted in an interview "Thought for food; the social and cultural aspects of malnutrition", by J. Balcomb. UNICEF News, 71: 6-12, 1972.

8. DEWALT, B.R. The illnesses no longer understand: changing conceptions of health and curing in a rural Mexican community. Med. Anthro. Newsletter, 8: 5-11, 1977.

9. DEWALT, B.R. Modernization in a Mexican Ejido. Cambridge University Press, Cambridge, 1979.

10. McFEE, M. The 150% man, a product of Blackfoot acculturation. Am. Anthro., 70: 1096-1103, 1968.

11. CLARK, M., KAUFMAN, S. and PIERCE, R. Explorations of acculturation: toward a model of ethnic identity. Human Org., 35: 231-238, 1976.

12. ROBERTS, F. Kenttätyö kokemuksia hämeestä. Suomen Antropologi, 3: 65-79, 1978.

13. HULL, C.H. and NIE, N.H. SPSS Update. McGraw Hill, New York, 1979.

NUTRITION RESEARCH, Vol. 1, pp. 73-82, 1981
0271-5317/81/010073-09$02.00/0 Printed in the USA.
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DIETARY CORN OIL DOES NOT SUPPRESS THE FRUCTOSE INDUCED INCREASE IN HEPATIC FATTY ACID SYNTHESIS

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ABSTRACT

The effect of dietary glucose and fructose on hepatic and whole body fatty acid synthesis in the presence and absence of added corn oil in the diet was examined in mice. Fructose-fed mice have higher rates of hepatic but not extra-hepatic fatty acid synthesis in vivo than do those fed glucose. In addition, fructose based diets elicit higher hepatic activities of fatty acid synthetase (FAS) ATP citrate lyase (CL), malic enzyme (ME) and glucose-6-phosphate dehydrogenase (G6PD) than do glucose based diets. Glucokinase (GK) was higher in glucose fed than fructose fed mice. Acetyl CoA carboxylase (AcCx) was the same on both diets. Inclusion of 5% corn oil in the diets decreased the activities of hepatic AcCx, FAS, ME, G6PD and in vivo hepatic and extra hepatic fatty acid synthesis in glucose fed mice but did not inhibit these in fructose fed mice except for G6PD where the inhibition was less than in glucose fed mice. Thus fructose feeding lead to higher rates of fat synthesis and less sensitivity to inhibition by dietary fat than does glucose feeding.

Key Words - Fructose; lipogenesis; corn oil, lipogenic enzymes.

INTRODUCTION

The role of fructose in lipid metabolism was suggested more than 60 years ago by Higgins (1) who concluded from measurements of respiratory quotients that fructose is more readily converted to lipid than is glucose. Since then, it has become apparent that the substitution of fructose for glucose in the diet of rats alters lipogenesis in liver and adipose tissue. It has been reported that the lipogenic enzymes including glucose-6-phosphate dehydrogenase (G6PD), malic enzyme (ME), ATP citrate lyase (CL), fatty acid synthetase (FAS) and in vivo fatty acid synthesis are increased in the liver of fructose fed rats (2-7). Further, rats eating a fructose based diet exhibit higher levels of serum triglycerides than those consuming glucose or starch (8). This fructose-induced hypertriglyceridemia may be associated with hyperinsulinemia (9).

The effects of fructose feeding are just the opposite in rat adipose tissue, that is, feeding fructose decreased G6PD, ME, Cl and FAS as well as extra hepatic fatty acid synthesis measured in vivo (3, 4, 5, 7).

Because these studies were generally conducted with animals allowed to feed ad libitum the possibility exists that the difference noted were due to differences in the intake of the experimental diets.

The addition of fat to fat-free diets has been shown to decrease the rate of lipogenesis (23). However, there are no reports of the effects of dietary fat on diets containing fructose in place of glucose.

This study was conducted to examine the effect of substituting fructose for glucose in diets with and without added corn oil on in vivo fatty acid synthesis in the liver and carcass of feed restricted mice. Further, the effects of these diets on hepatic levels of acetyl CoA carboxylase (AcCx), glucokinase (GK), FAS, CL, G6PD and ME were examined.

MATERIALS AND METHODS

Male mice C_{57} BL/6J (Jackson Labs, Bar Harbour, Maine) were individually housed in plastic metabolic cages. The animals were maintained at a temperature of ~25°C in a light controlled room (lights on 0800 - 2000). The animals were given 3.0g of food each day at 0900, water was available ad libitum. The diets were fed for a period of 14 days and had the composition indicated in Table 1. Six animals were assigned to each diet.

On the 14th day, the animals were injected intraperitoneally between 1000 and 1100 with 100μCi ^3H_2O and were decapitated 60 minutes later. At the time of sacrifice all animals had full stomachs. A sample of neck blood was taken for the determination of plasma specific radioactivity. The liver was rapidly removed, weighed and a portion homogenized in 3 volumes of cold 0.15M potassium chloride, 1.0mM magnesium chloride, 0.5mM dithiothreitol and 10mM N-acetyl cysteine buffer, pH 7.6. Following centrifugation at 100,000g for 40 min. the supernatant was used for quantitation of enzyme activities. Fatty acid synthetase was determined from the rate of malonyl CoA dependent NADPH oxidation (10), Glucose-6-phosphate dehydrogenase (E.C. 1.1.1.39, G6PD) from the rate of glucose-6-phosphate dependent reduction of NADP (11), malic enzyme (E.C. 1.1.1.40, ME) from the rate of malate dependent NADP reduction (12), glucokinase (E.C. 2.7.1.2, GK) from the rate of glucose dependent reduction of NADP in the presence of added glucose-6-phosphate dehydrogenase after correction of hexokinase activity (13), ATP citrate lyase (E.C. 4.1.3.8, CL) by the method of Srere (14) and acetyl CoA carboxylase from the acetyl CoA dependent incorporation of $H^{14}CO_3$ into acid stable material (15).

A second portion of the liver was used to determine fatty acid synthesis in vivo (16). The tissue was deposited directly into 5M potassium hydroxide and heated to 70° for 3 - 4 hours. After saponification, the samples were extracted with three 5ml portions of petroleum ether (b.p. 37.8-56.1°) to remove non-saponifiable material. The samples were then acidified with hydrochloric acid and the fatty acids extracted with three 5ml portions of petroleum ether. The extracts were combined in a scintillation vial and the radioactivity in the extracted fatty acids determined by liquid scintillation spectrometry. Protein was determined by the microbiuret method of Goa (17) using bovine serum albumin as standard.

Fatty acid synthesis in the extra hepatic tissues was determined as described by Baker et al. (19). Carcasses were saponified in 30% (50% ethanolic) KOH, 4ml/g carcass for 3 - 4 hours at 80°C. Non-saponfiable lipids were removed from the digests by three extractions with petroleum ether. After acidification with HCl the fatty acids were extracted with petroleum ether and after evaporation of the petroleum ether the fatty acids were assayed for ^3H by liquid scintillation spectrometry.

All chemicals and biochemicals were obtained from either Fisher Scientific Co. or Sigma Chemical Co.

Significance of treatment effects was determined by analysis of variance and Duncan's new multiple range test (18). A treatment was considered significant if $p < 0.05$.

TABLE 1

Composition of Diets
(g/100g)

	Glucose	Glucose & Corn Oil	Fructose	Fructose & Corn Oil
Casein	20	20	20	20
Glucose	70	65	-	-
Fructose	-	-	70	65
Corn Oil	-	5.0	-	5.0
Alpha Cellulose	5.0	5.0	5.0	5.0
AIN mineral mix[2]	3.5	3.5	3.5	3.5
AIN vitamin mix[2]	1.0	1.0	1.0	1.0
Choline Cl	0.2	0.2	0.2	0.2
Methionine	0.3	0.3	0.3	0.3

1. All diet components were purchased from ICN Pharmaceuticals, Cleveland, Ohio, except corn oil (Mazola™), which was obtained from Best Food Division, Canada Starch Co. Ltd., Montreal, Quebec.

2. Mixture 76™.

RESULTS

As shown in Table 2, during the two week feeding period the animals consumed the same amount of feed. Thus any differences observed can be attributed to the composition of the diets and not to differences in intake. The weight gain of both fructose-fed groups was greater than the glucose-fed animals although this did not reach statistical significance. This extra weight gain is almost entirely accounted for by the significantly greater liver weights in the fructose-fed animals. Inclusion of corn oil did not affect weight gain or liver weight in either glucose or fructose fed mice.

The results presented in Tables 3 and 4 were similar whether the results were expressed per gram liver or body weight or as total activity.

Acetyl CoA Carboxylase was present at the same level whether the mice consumed fructose or glucose-based diets however the inclusion of 5% corn oil decreased the activity of AcCx in glucose-fed mice but had no effect in fructose-fed mice. FAS was present at higher levels in fructose-fed mice. The lower levels observed in glucose-fed mice were further decreased by the inclusion of corn oil in the diet. Corn oil had no effect on the enzyme activity in fructose-fed mice. Its activity was not decreased by inclusion of corn oil in the diets. G6PD was higher in fructose than glucose-fed mice. With both dietary carbohydrates, inclusion of corn oil significantly inhibited the activity of G6PD.

The rate of lipogenesis in the extra-hepatic tissues was not increased by fructose feeding. However, as with hepatic synthesis inhibition was observed when corn oil was included with the glucose-based diet but not with the fructose-based diet.

DISCUSSION

We have confirmed earlier reports (2-7,19,20) that fructose feeding results in increased rates of hepatic fatty acid synthesis and the levels of several of the enzymes associated with fatty acid synthesis including fatty acid synthetase, glucose-6-phosphate dehydrogenase, malic enzyme and ATP citrate lyase. The finding of increased enzyme activity in the liver of fructose fed animals has not been universal. Michaelis and Szepsi (21) did not find any increase in G6PD in the liver of fructose-fed rats although ME was increased. Jeffcoat and James (22) did not observe any increase in hepatic FAS in rats fed diets containing 44% starch 20% fructose compared to 44% starch 20% glucose.

In mice fed glucose-based diets the inclusion of corn oil inhibits *in vivo* hepatic fatty acid synthesis and decreased the levels of FAS, G6PD and ME (23). However, the interaction of dietary fat and carbohydrate on hepatic lipogenesis has not been well studied. Carrol found the interactions to be complex (24, 25). For example substitution of fructose for glucose resulted in accumulation of less fat in livers of rats fed corn oil but of more fat in livers of rats fed hydrogenated oil. Since no measurements of fat synthesis or enzyme levels were made, it is not possible to determine whether these effects are due to increased synthesis, decreased export of VLDL or a combination. Our observation of increased liver size and decreased soluble protein/gram liver suggest that the livers of fructose-fed mice retained more fat than those of glucose-fed mice. While this may account for part of the increased incorporation of ^{3}H into liver fatty acids in fructose fed mice, it cannot explain the lack of inhibition by dietary corn oil. In addition, the increased lipogenic enzymes suggests that there was increased hepatic synthesis as well. Jeffcoat and James (22) reported that dietary corn oil (3%) is less

TABLE 2[1]

Liver weights, body weights, weight gain, feed consumption, and soluble liver protein

	DIETS			
	Glucose	Glucose + 5% Corn Oil	Fructose	Fructose + 5% Corn Oil
Liver weight	0.83 ± 0.11[a]	0.88 ± 0.06[a]	1.20 ± 0.14[b]	1.16 ± 0.17[b]
Initial body weight	20.06 ± 1.00	22.25 ± 1.11	20.92 ± 1.16	23.30 ± 0.87
Weight gain	0.93 ± 0.95	0.85 ± 0.44	1.48 ± 1.01	1.38 ± 0.85
Food consumed	39.52 ± 0.36	39.05 ± 1.02	40.38 ± 0.46	40.27 ± 0.59
Soluble protein	34.0 ± 3.86	32.0 ± 2.07	28.82 ± 2.56	29.67 ± 1.63

[1]All values are in grams except for soluble protein which is mg protein/ml 100,000 x g supernatant. Values are mean ± standard deviation. Means with different letter superscripts within the same row are significantly different ($p < 0.05$).

TABLE 3

Specific Activities of Hepatic Lipogenic Enzymes
and *In Vivo* Fatty Acid Synthesis

	DIETS			
	Glucose	Glucose + 5% Corn Oil	Fructose	Fructose + 5% Corn Oil
Acetyl CoA Carboxylase[1]	26.8 ± 7.43^{b}	15.3 ± 1.71^{a},[2]	26.9 ± 9.82^{b}	21.45 ± 6.90^{b}
Fatty Acid Synthetase	33.3 ± 6.18^{b}	24.8 ± 4.40^{a}	55.8 ± 10.08^{c}	48.05 ± 7.50^{c}
Citrate Cleavage Enzyme	52.68 ± 16.60^{b}	27.5 ± 7.15^{a}	97.57 ± 26.12^{d}	71.87 ± 20.95^{c}
Glucokinase	47.87 ± 6.62^{a}	43.93 ± 2.74^{a}	30.0 ± 4.10^{b}	29.83 ± 1.94^{b}
Malic Enzyme	209 ± 40^{b}	118 ± 21^{a}	347 ± 60^{c}	314 ± 48^{c}
Glucose-6-P Dehydrogenase	75.3 ± 20.5^{b}	29.67 ± 8.26^{a}	98.17 ± 24.18^{d}	49.83 ± 11.25^{c}
Liver Synthesis[3]	176.0 ± 59.1^{b}	118.4 ± 60.7^{a}	323.3 ± 67.3^{c}	327.0 ± 11.3^{c}
Carcass Synthesis[3]	57.7 ± 19.1^{b}	19.4 ± 12.1^{a}	61.2 ± 11.3^{b}	55.1 ± 9.1^{b}

[1] All enzyme activities are expressed as nmole/mg protein/min at 30°C.

[2] All values are mean ± standard deviation. Means with different letter superscripts within the same row are significantly different ($p < 0.05$).

[3] Synthesis is expressed as µgatoms ^{3}H from $^{3}H_2O$ incorporated into saponifiable fatty acids per gram tissue per hour.

TABLE 4

Total Activity of Hepatic Lipogenic Enzymes
and *In Vivo* Fatty Acid Synthesis

	Glucose	Glucose + 5% Corn Oil	Fructose	Fructose + 5% Corn Oil
Acetyl CoA Carboxylase[1]	3.02 ± 0.93	1.71 ± 0.22[a,2]	3.77 ± 1.64[b]	3.03 ± 1.36[b]
Fatty Acid Synthetase	4.41 ± 1.47[b]	2.82 ± 0.63[a]	7.72 ± 2.09[c]	6.70 ± 1.89[c]
Citrate Cleavage Enzyme	6.01 ± 2.17[b]	3.17 ± 1.04[a]	13.71 ± 5.19[c]	10.05 ± 3.79[c]
Glucokinase	5.45 ± 1.20[b]	4.96 ± 0.49[a,b]	4.19 ± 1.19[a]	4.08 ± 0.63[a]
Malic Enzyme	23.74 ± 6.03[b]	13.42 ± 3.10[a]	48.29 ± 14.42[c]	43.49 ± 10.25[c]
Glucose-6-P Dehydrogenase	8.63 ± 2.90[b]	3.38 ± 1.05[a]	13.70 ± 5.08[d]	6.95 ± 2.27[c]
Hepatic Synthesis[3]	155 ± 62[a]	107 ± 62[a]	389 ± 110[b]	398 ± 37[b]
Carcass Synthesis[3]	1130 ± 477[b]	424 ± 276[a]	1257 ± 201[b]	1282 ± 235[b]

[1]All enzyme activities are expressed as μmoles/liver/min at 30°C.

[2]All values are mean ± standard deviation. Means with different letter superscripts within the same row are significantly different ($p < 0.05$).

[3]Synthesis is expressed as μgatoms ^3H from ^3H_2O incorporated into saponifiable fatty acids for the entire liver or carcass per hour.

inhibitory when fructose rather than glucose is used to induce hepatic FAS. They found a 62% reduction in glucose + corn oil fed rats compared to a 46% reduction in those fed fructose + corn oil. Whether or not this is a significant difference cannot be determined from the results presented.

Our findings clearly demonstrate that dietary corn oil has no effect on the lipogenesis induced by fructose feeding nor with the exception of G6PD does it reduce the levels of the associated enzymes in liver. This is in contrast to the marked reductions observed when corn oil is included in the glucose based diet. G6PD was inhibited by corn oil in fructose-based diets. This is in contrast to ME which remains elevated. Thus it would appear that G6PD and ME are regulated differently under the conditions used. We have previously observed a dissociation between ME and G6PD in the livers of young rats fed varying protein levels which supports our view that the phosphogluconate pathway and ME, both involved in generation of NADPH are regulated by different mechanisms (G. R. Herzberg and M. Rogerson, unpublished observations).

We observed no increase in extra-hepatic fatty acid synthesis in fructose-fed rats however, as in the liver, the inclusion of corn oil did not decrease lipogenesis in fructose based diets as it did in those containing glucose.

If the rates of synthesis in the entire animal (liver and carcass) are examined it is apparent that not only is the capacity increased in fructose fed mice compared to those fed glucose but there is no reduction in synthesis when corn oil is included in the fructose-based diets while the synthetic rate is diminished approximately 50% by corn oil in the glucose-based diets. Further, a comparison of the site of synthesis shows that in the fructose-fed mice approximately one-third of the synthesis is hepatic while when glucose is fed only 15 - 20% occurs in the liver. Thus, fructose feeding results in enhanced rates of fatty acid synthesis as well as a redistribution between the sites of synthesis.

The mechanism for the lack of inhibition of fructose-induced lipogenesis remains unclear. One possibility is that the inhibitory effect of dietary fat in glucose-based diets is insulin mediated. This hypothesis is complicated by the varying reports of the effect of feeding fructose compared to glucose on plasma insulin. For example, it has been generally reported that in rats fructose feeding results in lowered plasma insulin (4, 26). However, a recent report showed no differences in serum insulin concentrations at any time of day between rats fed glucose or fructose (8). Finally, Reaven (9) recently reported a rise in plasma insulin from 55μU/ml in rats fed glucose to 85μU/ml in those fed fructose. It would therefore appear that the conclusion of Jeffcoat and James (22) that the major inhibitory effect of dietary corn oil cannot be explained by a mechanism which involved control of insulin is not warranted at this time.

In conclusion, we have demonstrated that fructose feeding leads to increased lipogenesis in mice and that this enhanced synthesis is not inhibited by inclusion of corn oil in the diet as it is in diets containing glucose.

ACKNOWLEDGEMENTS

Research was supported in part by grants from the Medical Research Council of Canada (MA-6097) and from the Banting Research Foundation. We wish to thank Dr. S. Mookerjea for helpful discussions.

REFERENCES

1. HIGGINS, H.L. The rapidity with which alcohol and some sugars may serve as nutrients. Am. J. Physiol., 41: 258-265, 1916.

2. FIRCH, W.M. and CHAIKOFF, I.L. Extent and patterns of enzyme activities in livers of normal rats fed diets high in glucose and fructose. J. Biol. Chem., 235: 554-557, 1969.

3. CHEVALIER, M.M., WILEY, J.H. and LEVEILLE , G.A. Effect of dietary fructose on fatty acid synthesis in adipose tissue and liver of the rat. J. Nutr., 102: 337-342, 1972.

4. BROCKDORFER, K.R., KHAN, I.H. and YUDKIN, J. Fatty acid synthetase activity in the liver and adipose tissue of rats fed with various carbohydrates. Biochem. J., 129: 239-446, 1972.

5. NAISMITH, D.J. Differences in the metabolism of dietary carbohydrates studied in the rat. Proc. Nutr. Soc., 30: 259-265, 1971.

6. COHEN, A.M., BRILLER, S. and SHAFIR, E. Effect of long term sucrose feeding on the activity of some enzymes regulating glycolysis, lipogenesis and gluconeogenesis in rat liver and adipose tissue. Biochem. Biophys. Acta, 279: 129-138, 1962.

7. ROMSOS, D.R. and LEVEILLE, G.A. Effect of dietary fructose on in vitro and in vivo fatty acid synthesis in the rat. Biochem. Biophys. Acta, 360: 1-11, 1974.

8. MERTENS, L.S., TEPPERMAN, H.M. and TEPPERMAN, J. Effect of short-term dietary glucose and fructose on rat serum tryglyceride concentration. J. Nutr., 110: 982-998, 1980.

9. SLEDER, J., CHEN, Y.I., CULLY, M.D. and REAVEN, G.M. Metabolism, 29: 303-305, 1980.

10. GIBSON, D.M. and HUBBARD, D.D. Incorporation of malonyl CoA into fatty acids by liver in starvation and alloxan-diabetes. Biochem. Biophys. Res. Comm., 3: 531-535, 1960.

11. LOHR, G.W. and WALLER, H.D. Methods of enzymatic analysis (H.V. Bergmeyer, Ed.). Academic Press, New York, 1971, Vol, II, pp. 636.

12. YEH, Y.Y., LEVEILLE, G.A. and WILEY, J.H. Influence of dietary lipid on lipogenesis and on the activity of malic enzyme and citrate cleavage enzyme in liver of the growing chick. J. Nutr., 100: 917-924, 1970.

13. PILKIS, S.J. Glucokinase in rat liver. In: Methods of Enzymology. W.A. Wood (ed). Academic Press, New York, 1975, Vol. 42, pp. 31-39.

14. SRERE, P.A. The citrate cleavage enzyme. J. Biol. Chem., 234: 2544-2547, 1959.

15. GOVE, C.D., and HEMS, D.A. Fatty acid synthesis in the regenerating liver of the rat. Biochem. J., 170: 1-8, 1978.

16. LOWENSTEIN, J.M. Effect of (-)-hydroxycitrate on fatty acid synthesis by rat liver in vivo. J. Biol. Chem., 246: 629-632, 1971.

17. GOA, J. Micro Biuret Method for protein determination: Total protein in cerebrospinal fluid. Scand. J. Clin. Lab. Invest., 5: 218-222, 1953.

18. STEEL, R.G.D. and TORRIE, J.H. Principles and Procedures of statistics. McGraw-Hill Book Company, New York, 1960.

19. BAKER, N., LEARN, D.B. and BRUCKDORFER, K.R. Re-evaluation of lipogenesis from dietary glucose carbon in liver and carcass of mice. J. Lipid Res., 19: 879-893, 1978.

20. VOLPE, J.J. and VAGELOS, P.R. Regulation of mammalian fatty-acid synthetase. The roles of carbohydrate and insulin. Proc. Natl. Acad. Sci. U.S.A., 71: 889-893, 1974.

21. MICHAELIS, O.E. and SZEPESI, B. The mechanism of a specific metabolic effect of sucrose in the rat. J. Nutr. 104: 1597-1609, 1974.

22. JEFFCOAT, R. and JAMES, A.T. Interrelationship between the dietary regulation of fatty acid synthesis and the fatty acyl-CoA desaturases. Lipids, 12: 469-474, 1977.

23. HERZBERG, G.R. and JANMOHAMMED, N. Regulation of hepatic lipogenesis by dietary maize oil or tripalmitin in the meal-fed mouse. Brit. J. Nutr., 43: 571-579, 1980.

24. CARROLL, C. Influences of dietary carbohydrate-fat combinations on various functions associated with glycolysis and lipogenesis in rats. J. Nutr., 82: 163-172, 1964.

25. CARROLL, C. and BRIGHT, E. Influence of carbohydrate-to-fat ratio on metabolic changes induced in rats by feeding different carbohydrate-fat combinations. J. Nutr., 87: 202-210, 1965.

26. CRYER, A., RILEY, S.E., WILLIAMS, E.R. and ROBINSON, D.S. Effects of fructose, sucrose and glucose feeding on plasma insulin concentrations and on adipose-tissue clearing-factor lipase activity in the rat. Biochem. J., 140: 561-563, 1974.

NUTRITION RESEARCH, Vol. 1, pp. 83-92, 1981
0271-5317/81/010083-10$02.00/0 Printed in the USA.
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CHARACTERISTIC PROPERTIES OF ISOLATED SOY PROTEIN IN THE METABOLIC CHANGES DUE TO DIETARY POLYCHLORINATED BIPHENYLS

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ABSTRACT

Feeding polychlorinated biphenyls (PCB) to rats causes an increase in activity of liver microsomal drug-metabolizing enzyme system, plasma level of cholesterol and urinary ascorbic acid. The influences of different types of dietary protein, including whole-egg protein, isolated soy protein and wheat gluten, on these metabolic responses were studied. In the animals not receiving PCB, the activity of drug-metabolizing enzymes and plasma cholesterol were well correlated with the quality of dietary protein as shown by growth rate and nitrogen balance. In the animals fed 200 ppm of PCB containing diets, aniline hydroxylase activity still correlated with the protein quality, but in the animals fed isolated soy protein diets containing PCB, aminopyrine N-demethylase activity, cytochrome P-450 content, plasma cholesterol and urinary ascorbic acid were less than those expected from the biological value. However, supplementation of methionine to isolated soy protein diet containing PCB caused a marked increase in urinary ascorbic acid and plasma cholesterol. This suggests that more sulfur-containing amino acids are required for the maximum increase in these metabolic parameters than those for the growth rate and nitrogen balance.

KEY WORDS: PCB, protein quality, sulfur-containing amino acids, drug-metabolizing enzymes, plasma cholesterol, urinary ascorbic acid.

INTRODUCTION

We have shown before that the rats exposed to PCB exhibited an increase in the activity of hepatic drug-metabolizing enzymes, plasma cholesterol and urinary ascorbic acid (1,2). Similar observation have been reported with other xenobiotics, including phenobarbital, pentobarbital, caffein, 2,6-di-tert-butyl-*p*-cresol (BHT) and 1,1,1-trichloro-2,2 bis (*p*-chlorophenyl)ethane (DDT), in which a general correlation between those metabolic responses was observed (2,3). Studies with rats fed PCB containing diets demonstrated an induction of hepatic cholesterol synthesizing system, including

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3-hydroxy-3-methylglutaryl coenzyme A reductase (4). Ascorbic acid plays some protective role against the exposure to the chemicals since massive amount of dietary ascorbic acid ameliorated the toxicity of PCB in guinea pigs (5). The increase in blood cholesterol due to the chemicals might be in part relating to the enhanced production of corticosterone in adrenal under such stress.

Recently it was found in our laboratory that the increase in plasma cholesterol and urinary ascorbic acid due to PCB intake was greatly affected with dietary level of protein (1,6). This report deals with influence of protein quality on these metabolic responses to PCB.

METHODS

Experiment 1

Male rats of the Wistar strain weighing 59-85g were used. All rats were housed in stainless steel cages with raised wire floors under controlled lighting (0800-2000 hours) and temperature 24°C. These rats were maintained on the test diets which were composed of 10% protein, 0.5% vitamin mixture (7) without ascorbic acid, 5% salt mixture (7), 5% corn oil, 4% cellulose powder and 0.2% choline chloride. Retinyl acetate, cholecalciferol, DL-α-tocopherylacetate and sodium selenite were added at the level of 10,000 IU, 4000 IU, 100 mg and 0.2 mg per Kg diet, respectively. As the test protein, whole-egg protein (Taiyo Food Co. Ltd., Yokkaichi, Japan), isolated soy protein (Ajipron 90A, Ajinomoto Co. Ltd., Tokyo) and wheat gluten (Esaki Glico Co. Ltd., Osaka) were used at the expense of carbohydrate composed of one part of sucrose and two parts of gelatinized corn starch. PCB (Aroclor 1248, Mitsubishi Monsant Co. Ltd., Tokyo) was added to the diets at the level of 200 ppm. Nitrogen content of each protein was determined by Kjeldahl method. After feeding the test diet for 30-32 days, the animals were anesthetized with ether and killed at 1300 hour within a 60-minutes period. Blood was collected by heart puncture in heparinized syringes for plasma lipid analysis. The livers were then immediately removed, weighed and used for enzyme analysis.

Liver homogenate was prepared with ice-cold 1.15% KCl in 0.01M phosphate buffer, pH 7.4 with a Potter-Elvejem homogenizer. The crude homogenate was spun at 10,000×g for 10 minutes, and the postmitochondrial supernatant was used for the enzyme assays. Microsomes were prepared by spinning an aliquot of each pool of 10,000×g for 1 hour at 100,000×g. The pellet was suspended in 0.05M of phosphate buffer containing 0.001 M EDTA and assayed for microsomal protein and cytochrome P-450. Cytochrome P-450 was measured by the method of Omura and Sato (18). Microsomal protein was determined by the procedure of Lowry et al. (9).

The assay system for aminopyrine N-demethylase consisted of 2 ml of the postmitochondrial supernatant (correspond to 250 mg of liver), 1 ml of aminopyrine 5 μmoles and $MgCl_2$ 25

TABLE 1

Effects of Dietary Addition of 200 ppm PCB and Dietary Protein Quality on Gains in Body Weight and Tissue Weights[1]

Groups	Gains in body wt.	Tissue wt. (per 100g body wt.)		
		Liver	Spleen	Adrenal
	g/30 days	g	mg	mg
Normal				
whole egg protein	117.2 ± 3.5[2]	3.86 ± 0.08	185 ± 9	11.7 ± 0.4
isolated soy protein	43.8 ± 4.2	4.06 ± 0.05	165 ± 2	13.5 ± 0.4
wheat gluten	25.0 ± 3.5	3.98 ± 0.10	174 ± 8	14.5 ± 0.4
PCB				
whole egg protein	121.8 ± 3.9	5.55 ± 0.16	196 ± 7	11.9 ± 0.3
isolated soy protein	39.0 ± 1.8	6.24 ± 0.20	172 ± 5	13.3 ± 0.3
wheat gluten	18.2 ± 1.2	5.77 ± 0.12	165 ± 9	15.7 ± 0.7

1. Initial body weight, average 77.2-77.5g (range 59-85g). Experimental period 30-32 days. 2. Mean ± SEM (N = 6).

TABLE 2

Effects of Dietary Addition of PCB and Dietary Protein Quality on Nitrogen Balance and Urinary Excretion of Ascorbic Acid

Groups	Nitrogen balance		Urinary ascorbic acid	
	on day 15-16	on day 25-26	on day 15-16	on day 25-26
	mg nitrogen / day		mg/100g body wt./day	
Normal				
whole egg protein	144 ± 11[1]	131 ± 7	0.28 ± 0.02	0.34 ± 0.04
isolated soy protein	54.6 ± 8.7	53.1 ± 4.3	0.11 ± 0.04	0.11 ± 0.02
wheat gluten	8.0 ± 3.3	21.2 ± 1.4	0.19 ± 0.04	0.12 ± 0.02
PCB				
whole egg protein	134 ± 12	148 ± 7	12.8 ± 0.4	12.1 ± 0.6
isolated soy protein	39.9 ± 3.5	45.9 ± 5.1	0.95 ± 0.18	1.49 ± 0.19
wheat gluten	7.2 ± 4.0	17.3 ± 7.0	5.36 ± 0.19	6.45 ± 0.53

1. Mean ± SEM (N = 6).

μmoles and 3 ml of 0.5 M phosphate buffer, pH 7.4 containing NADP 0.65 μmoles, glucose-6-phosphate 10 μmoles, nicotinamide 50 μmoles and semicarbazide 45 μmoles. The assay system for aniline hydroxylase consisted of 1 ml of the postmitochondrial supernatant (correspond to 250 mg of liver) and 3 ml of 0.1 M phosphate buffer, pH 7.4, containing aniline HCl 5 μmoles, $MgCl_2$ 25 μmoles, NADP 0.5 μmoles, glucose-6-phosphate 10 μmoles and nicotinamide 50 μmoles. The mixture was incubated for 12 minutes at 37°C. The N-demethylation of aminopyrine was stopped by the addition of 2 ml of 15% $ZnSO_4$, followed by 2 ml of saturated solution of $Ba(OH)_2$. The hydroxylation of aniline was stopped by the addition of 2 ml of 20% trichloroacetic acid. The N-demethylation of aminopyrine was determined by measuring the liberated formaldehyde according to the method of Nash (10). The hydroxylation of aniline was estimated by measuring the amount of *p*-aminophenol formed (2).

Urine was collected into 15 ml of 5% metaphosphoric acid solution and centrifuged at 5000×g. A portion of the tissue was homogenized with 5% metaphosphoric acid solution and then centrifuged. Ascorbic acid in the metaphosphoric acid extracts of tissues and urine was determined by the 2,4-dinitrophenylhydrazine method (11).

Liver total lipids were extracted by the method of Folch et al.(12) and used for the determination of cholesterol. Cholesterol in plasma and liver was measured by the method of Pearson et al. (13). Plasma triglyceride was determined with a commercial kit (E-CG01, Eiken Chemical Co. Ltd., Tokyo).

Experiment 2

Rats weighing 98-118g were fed the test diets containing 200 ppm of PCB for 18 days. The diets contained either isolated soy protein, isolated soy protein plus 0.34% L-methionine or wheat gluten fed at 10% of the diet. Other dietary composition was the same as experiment 1. Urinary and liver ascorbic acid and plasma cholesterol were determined.

The data were analyzed statistically by analysis of variance and the means compared using Duncan's multiple range test (14).

RESULTS

Effects of dietary addition of 200 ppm PCB and dietary protein on gains in body weight, nitrogen balances and tissue weight were indicated in table 1 and 2. Dietary PCB at this level caused no significant effect on gains in body weight, nitrogen balances and weights of spleen and adrenal. However, the enlargement of the liver due to PCB was observed. Adrenal weights per 100g body weight correlated inversely with the quality of protein as shown by gains in body weight and nitrogen balances.

A marked increase in urinary ascorbic acid due to PCB intake was observed (table 2). Particulary, this increase was striking in whole-egg protein and wheat gluten (28-54 fold increases). Isolated soy protein diet permitted less increment (9-14 fold). As shown in table 3, dietary PCB caused an

TABLE 3

Effect of Dietary Addition of PCB and Dietary Protein Quality on Tissue levels of ascorbic acid

Groups	Liver	Spleen	Adrenal
		mg / g tissue	
Normal			
whole egg protein	0.237 ± 0.012[a1]	0.515 ± 0.009[a]	4.75 ± 0.16[ad]
isolated soy protein	0.137 ± 0.007[b]	0.339 ± 0.005[b]	3.37 ± 0.05[b]
wheat gluten	0.205 ± 0.005[a]	0.440 ± 0.016[c]	4.04 ± 0.13[c]
PCB			
whole egg protein	0.720 ± 0.016[c]	0.650 + 0.014[d]	5.02 ± 0.11[a]
isolated soy protein	0.448 ± 0.018[d]	0.580 ± 0.013[e]	4.67 ± 0.09[d]
wheat gluten	0.565 ± 0.013[e]	0.571 ± 0.011[e]	4.77 ± 0.08[ad]

1. Mean ± SEM. (N = 6). Means within a column not followed by the same letter are significantly different ($P < 0.05$).

TABLE 4

Effect of Dietary Addition of PCB and Dietary Protein Quality on Plasma and Liver Lipids

Groups	Plasma cholesterol	Plasma triglyceride	Liver cholesterol
	mg/100 ml	mg/100 ml	mg/g tissue
Normal			
whole egg protein	97 ± 3[abl]	248 ± 38[a]	3.65 ± 0.17[a]
isolated soy protein	89 ± 3[a]	46 ± 4[b]	2.97 ± 0.29[c]
wheat gluten	74 ± 3[c]	23 ± 2[c]	3.17 ± 0.18[bcd]
PCB			
whole egg protein	153 ± 6[d]	246 ± 24[a]	3.89 ± 0.19[a]
isolated soy protein	107 ± 3[b]	35 ± 5[d]	2.83 ± 0.08[cd]
wheat gluten	125 ± 6[e]	50 ± 3[b]	3.26 ± 0.28[bd]

1. Mean ± SEM (N = 6). Means within a column not followed by the same letter are significantly different ($P < 0.05$).

increase in tissue concentration of ascorbic acid. Tissue ascorbic acid in soy protein groups was also somewhat less than those of other protein diets regardless of PCB addition.

Effects of PCB and protein quality on plasma and liver lipids were indicated in table 4. PCB intake increased plasma cholesterol. Although plasma levels of cholesterol in normal groups were well correlated with the protein quality, plasma levels of cholesterol in PCB groups were not so well correlated, since plasma cholesterol in rats fed soy protein diet containing PCB was significantly less than those in gluten diets containing the chemical ($P<0.05$). Liver concentration of cholesterol in the soy protein diets was also slightly less than those in other protein diets regardless of PCB addition. The gluten diet permitted a significant increase ($P < 0.05$) in plasma triglyceride due to PCB, but not other protein diets.

Effects of PCB and protein on liver microsomal drug-metabolizing enzyme system were presented in table 5. Enzyme activities were expressed both per g liver and per 100g body weight. Dietary PCB caused a significant increase in the activity of aminopyrine N-demethylase, aniline hydroxylase and cytochrome P-450 level ($P<0.05$). In normal groups not receiving PCB, the activities of the enzyme system correlated well with protein quality as shown by growth rates. In PCB groups, the activity of aniline hydroxylase was also well correlated with the protein quality. Aminopyrine demethylase activity, cytochrome P-450 level and microsomal protein level in the soy protein diets containing PCB were, however, less than those in gluten diets containing the chemical.

In the second experiment, the influence of supplementation of methionine to isolated soy protein diet containing PCB was studied. Data on gains in body weight, urinary and liver ascorbic acid and plasma cholesterol were presented in table 6. Supplementation of methionine to the soy protein diet resulted in a remarkable increase in the growth rate and urinary ascorbic acid. Liver concentration of ascorbic acid and plasma cholesterol were also significantly increased by the supplementation of methionine ($P < 0.05$). In addition, the values of these metabolic parameters in the soy protein diet supplemented with methionine were higher than those in wheat gluten diet.

DISCUSSION

It has been reported that exposure of rats to xenobiotics results in an increase in the activity of liver microsomal drug-metabolizing enzyme system, which is affected by dietary level of protein (15,18). Recently it was found in our laboratory that dietary addition of PCB caused an increase in urinary ascorbic acid and plasma level of cholesterol, which was greatly potentiated with high protein diet (6). The present study provided evidence that quality of protein also influenced these metabolic responses.

In the animals not receiving PCB, activities of drug-

TABLE 5

Effect of Dietary Addition of PCB and Dietary Protein Quality on the Activity of Liver Microsomal drug-metabolizing enzymes

	Normal			PCB		
	Whole egg protein	Isolated soy protein	Wheat gluten	Whole egg protein	Isolated soy protein	Wheat gluten
Aminopyrine demethylase						
unit[1]/g liver	1.03 ± 0.11[a4]	0.40 ± 0.05[bd]	0.29 ± 0.03[b]	3.15 ± 0.21[c]	0.66 ± 0.11[d]	1.23 ± 0.15[a]
unit[1]/100g body wt.	4.65 ± 0.08[a]	1.60 ± 0.18[b]	1.14 ± 0.22[b]	17.51 ± 1.28[c]	4.01 ± 0.53[a]	7.15 ± 0.97[d]
Aniline hydroxylase						
unit[2]/g liver	119 ± 7[a]	82 ± 7[ab]	58 ± 4[b]	524 ± 17[c]	288 ± 15[d]	274 ± 25[d]
unit[3]/100g body wt.	0.45 ± 0.03[a]	0.33 ± 0.03[ab]	0.23 ± 0.01[b]	2.92 ± 0.12[c]	1.79 ± 0.08[d]	1.59 ± 0.16[d]
Cytochrome P-450						
nmoles/g liver	18.5 ± 0.7[a]	9.9 ± 0.8[b]	9.8 ± 0.6[b]	62.5 ± 2.9[c]	27.3 ± 1.5[d]	32.6 ± 2.0[e]
nmoles/100g body wt.	71 ± 3[a]	40 ± 3[b]	39 ± 2[b]	348 ± 22[c]	170 ± 8[d]	188 ± 13[d]
Microsomal protein						
mg/g liver	15.5 ± 0.3[a]	9.7 ± 0.8[b]	10.1 ± 0.4[b]	21.9 ± 0.6[c]	11.6 ± 1.0[d]	14.9 ± 0.8[a]
mg/100g body wt.	59.9 ± 1.6[a]	39.6 ± 3.7[b]	39.9 ± 0.6[b]	126.1 ± 6.1[c]	76.4 ± 5.8[d]	85.9 ± 5.5[d]

1. Unit of enzyme activity was expressed as μmoles of formaldehyde produced per 12 minutes.
2. Unit of enzyme activity was expressed as nmoles of *p*-aminophenol produced per 12 minutes.
3. Unit of enzyme activity was expressed as μmoles of *p*-aminophenol produced per 12 minutes.
4. Mean ± SEM (N = 6). Means within a column not followed by the same letter are significantly different ($P < 0.05$).

TABLE 6

Effect of Supplementation of Methionine to Isolated Soy Protein on Gains in Body Weight, Urinary Ascorbic Acid and Plasma Cholesterol in Rats fed 200 ppm PCB containing Diets[1]

Groups	Gains in body wt.	Urinary ascorbic acid	Liver ascorbic acid	Plasma cholesterol
	g/18 days	mg/100g body wt/day[3]	µg/g tissue	mg/100 ml
isolated soy protein	37.8 ± 2.8[2]	1.44 ± 0.25	524 ± 18	112 ± 4
isolated soy protein + 0.34% methionine	85.3 ± 2.5	9.38 ± 0.66	785 ± 33	144 ± 3
wheat gluten	17.3 ± 2.5	3.46 ± 0.20	637 ± 29	122 ± 5

1. Initial body weight, average 111.6g (range 98-118g). Experimental period 18 days.
2. Mean ± SEM (N = 6). 3. on day 12-13.

metabolizing enzyme system and plasma cholesterol were well correlated with the protein quality. In the animals received PCB, the activity of aniline hydroxylase was still well correlated with the protein quality. In accordance with these results, Miranda and Webb (19) reported that higher activities of drug-metabolizing enzymes were obtained with rats fed casein diet supplemented with methionine as compared with those fed gluten diets, where a positive correlationship between the biological values and the enzyme activities was observed. However, it was found that liver aminopyrine demethylase activity and cytochrome P-450 content in the isolated soy protein group receiving PCB in our experiment were significantly less than those in the gluten group receiving PCB, although the quality of the soy protein was higher than that of wheat gluten, as evidenced by the growth rate and nitrogen balance. It was also found that the increase in urinary ascorbic acid and plasma cholesterol by PCB was appreciably less in the isolated soy protein group than that expected from the biological value. This finding suggests that the isolated soy protein diet might be deficient in some factors permitting those metabolic responses to PCB. Dietary levels of sulfur-containing amino acids in whole-egg protein diet, isolated soy protein diet and wheat gluten diet are 0.58, 0.28 and 0.40%, respectively, which well correlated with the metabolic responses on each diet to PCB. Therefore, we postulated that the factors just mentioned might be possibly these amino acids. This view was supported by the second experiment indicating the marked increase in urinary ascorbic acid and plasma cholesterol by the supplementation of methionine to the soy protein diet containing PCB. In this experiment, the dietary levels of sulfur-containing amino acids in soy protein diet, soy protein plus methionine diet and wheat gluten diet were 0.28, 0.62 and 0.40%, respectively, which positively correlated with the metabolic changes (table 6). Sulfur-containing amino acids are of special importance in the animals receiving xenobiotics since glutathione and sulfuric acid derived from the amino acids are used for

conjugation of metabolites of xenobiotics. The present finding may suggest a new aspect of the role of sulfur-containing amino acids in the adaptation to exposure to xenobiotics.

Magdalou et al. (20) reported that supplementation of sulfur-containing amino acids to the diet slightly decreased liver microsomal cytochrome P-450 content. Fielding and Hughes (21) also reported a trend in the decrease in liver level of cytochrome P-450 by supplementation of methionine to 20% soy protein without xenobiotics. These studies and the present result obtained here were apparently contradictory. It is considered that this is due to the presence of xenobiotics, PCB in the diets, but further study is necessary to clarily the discrepancy.

REFERENCES

1. Kato, N., Kato, M., Kimura, T. and Yoshida, A. Effect of dietary addition of PCB, DDT or BHT and dietary protein on vitamin A and cholesterol metabolism. Nutr. Rep. Inter., 18: 437-445, 1978.

2. Kato, N. and Yoshida, A. Effects of some fat-soluble chemicals on plasma cholesterol and urinary ascorbic acid in rats. Agric. Biol. Chem., 43: 191-192, 1979.

3. Magdolna, K., Robert, T., Magdolna, B., Erns, D., Magda, T. K. and Eva, W. J. Effect of caffeine and barbiturates on the metabolism of rats. Egeszsegutudomany, 13: 293-300, 1969.

4. Kato, N. and Yoshida, A. Effect of dietary PCB on hepatic cholesterogenesis in rats. Nutr. Rep. Inter., 21: 107-112, 1980.

5. Kato, N., Okada, T., Takenaka, Y. and Yoshida, A. Ameliorative effect of dietary ascorbic acid on PCB toxicity in guinea pigs. Nutr. Rep. Inter., 15: 125-130, 1977.

6. Kato, N., Tani, T. and Yoshida, A. Effect of dietary level of protein on liver microsomal drug-metabolizing enzymes, urinary ascorbic acid and lipid metabolism in rats fed PCB-containing diets. J. Nutr., 110: 1686-1694, 1980.

7. Harper, A. E. Amino acid balance and imbalance. 1. Dietary level of protein and amino acid imbalance. J. Nutr., 68: 405-418, 1959.

8. Omura, T. and Sato, R. The carbon monoxide binding pigment of liver microsomes. J. Biol. Chem., 239: 2370-2378, 1964.

9. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall,

R. J. Protein measurement with Folin phenol reagent. J. Biol. Chem., 193: 265-275, 1951.

10. Nash, T. The colorimetric estimation of formaldehyde by means of the Hantzch reaction. Biochem. J., 55: 416-421, 1953.

11. Ohara, T. Shokuhinbunsheki Handbook, pp 287-289, Kenpaku Co., Tokyo, 1973.

12. Folch, J., Lees, M. and Sloane-Stanley, G. H. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem., 226: 497-509, 1957.

13. Pearson, S., Stern, S. and McGavack, T. H. A rapid, accurate method for the determination of total cholesterol in man. Anal. Chem., 25: 813-814, 1953.

14. Duncan, D. B. Multiple range and multiple F test. Biometrics, 11: 1-6, 1955.

15. McLean, A. E. and McLean, E. K. The effect of diet and 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl) ethane (DDT) on microsomal hydroxylating enzymes and sensitivity of rats to carbon tetrachloride poisoning. Biochem. J., 100: 564-571, 1969.

16. Marshall, W. J. and McLean, A. E. M. The effect of oral phenobarbitone on hepatic microsomal cytochrome P-450 and demethylation activity in rats fed normal and low protein diets. Biochem. Pharmacol., 18: 153-157, 1969.

17. Hayes, J. R., Mgbodile, M. U. K. and Campbell, T. C. Effect of protein deficiency on the inducibility of the hepatic microsomal drug-metabolizing enzyme system. I. Biochem. Pharmacol., 22: 1005-1014, 1973.

18. Magbodile, M. U. K., Hayes, J. R. and Campbell, T. C. Effect of protein deficiency on the inducibility of the hepatic microsomal drug-metabolizing enzyme system. II. Biochem. Pharmacol., 22: 1125-1132, 1973.

19. Miranda, C. L. and Webb, R. E. Effects of dietary protein quality on drug metabolism in the rats. J. Nutr., 103: 1425-1430, 1973.

20. Mgdalou, J., Steimetz, D., Batt, A., Poullain, B., Siest, G. G. and Debry, G. The effect of dietary sulfur-containing amino acids on the activity of drug-metabolizing enzymes in rat liver microsomes. J. Nutr., 109: 864-871, 1979.

21. Fielding, A. M. and Hughes, R. E. Changes in liver microsomal cytochrome P-450 induced by dietary proteins and lipid material. Biochem. Pharmacol., 25: 1916-1917, 1976.

NUTRITION RESEARCH, Vol. 1, pp. 93-99, 1981
0271-5317/81/010093-07$02.00/0 Printed in the USA.
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SERUM CARNITINE LEVELS AND HEPATIC AND ADIPOSE TISSUE CARNITINE TRANSFERASES IN OBESE MICE

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ABSTRACT

Plasma levels of total and acylcarnitine were lower in young obese (ob/ob) mice than in their lean litter mates. However, the activities of carnitine-acetyl- and carnitine-palmitoyl transferases (CAT and CPT) were higher in the obese animals. CAT in brown fat of obese mice was also elevated. Fatty acid synthetase (FAS) and phosphoenolpyruvate carboxykinase (PEPcK) in brown fat were lower in activity in obese than in lean mice.

Key words: obesity, carnitine, carnitine transferases, fatty acid synthetase.

INTRODUCTION

It has been observed that obese mice have difficulties in maintaining their body temperature (1) and this has been related to a deficiency in the oxidation of fatty acids and impaired function of brown adipose tissue (2).

Carnitine is known to be essential for optimum fatty acid utilization by mitochondria (3) and we have shown it to be particularly important in heat production in brown adipose tissue (4). We have also shown that increased fat utilization (starvation, high fat diet) leads to elevated levels of acylcarnitine in the plasma (5, 6).

In the experiments reported below, we compared ob/ob mice and their lean litter mates with the aim to throw some light on the possible role of carnitine in lipid utilization by obese mice. We determined free and total plasma carnitine, hepatic carnitine acetyltransferase (CAT) and carnitine palmitoyl transferase (CPT).

In addition, activities of fatty acid synthetase (FAS) and phosphoenolpyruvate carboxykinase (PEPcK) were also assayed, so as to obtain a more general picture of fat and carbohydrate metabolism in young obese mice.

MATERIALS AND METHODS

Several male and female mice of the C57BL/67 strain (ob/+) were obtained from Jackson Laboratories, Bar Harbour, Maine, U.S.A., and these were bred in our laboratory. Usually litters of 6 to 9 animals contained one to three obese mice. These could first be visually recognized when aged 4 to 6 weeks and were used at that age, or soon after.

Male obese mice and their litter mates were always sacrificed in the morning of the same day. After decapitation, blood was collected into heparinized tubes and tissues were rapidly removed and placed on ice. The liver was divided into two parts (after removal of the gall bladder); one part served for CAT and CPT determinations, the other for FAS and PEPcK determinations. Brown adipose tissue was taken from between the shoulder blades and white adipose tissue was epidydimal in origin.

For CAT (E.C. 2.3.1.7) and CPT (2.3.1.21) determinations livers were homogenized in 0.25 M sucrose, 1mM EDTA, and 3.4mM HEPES, pH 7.5, using a teflon pestle and glass homogenizer. Mitochondria were isolated after a preliminary spin at 2,000 rpm by centrifugation at 8,000 x g for 15 minutes at 4°C and then were resuspended in the original buffer. CAT and CPT activities were assayed as described previously (7, 8). For FAS determination, tissues were homogenized in 0.25M sucrose, 1mM EGTA and 3.4mM TRIS, pH 7.4 and spun at 105,000 x g for 45 minutes at 4°C in a Beckman ultra-centrifuge (LS 85). FAS was assayed according to Volpe et al (9) by measuring the rate of oxidation of NADPH; and PEPcK (E.C. 4.1.1.32) according to Hopgood et al (10). Plasma carnitine (free and total) and β-hydroxybutyrate were determined as described previously (5). The difference between total and free carnitine was taken to be acylcarnitine (i.e. all water soluble esters of carnitine). Protein was assayed according to Lowry et al (11).

RESULTS

Plasma levels of total carnitine and acylcarnitine were significantly lower in obese mice than in their lean litter mates (Table I). However, there was no statistically significant difference in the levels of β-hydroxybutyrate between the two groups. The activity of hepatic CAT and CPT was higher in obese than in lean mice (Table I). As reported by others (12) hepatic FAS activity was higher in the obese than in the lean group. No difference was found between the two groups in the activity of PEPcK in the liver.

TABLE I

Plasma Levels of Carnitine and Hepatic Enzyme Activities in Lean and Obese Mice Aged 50 to 70 Days

	Obese	Lean
Body Weight, g[b]	35.2 ± 2.2	24.7 ± 2.12
Serum β-hydroxybutyrate, mmoles/ℓ	0.143 ± 0.03	0.39 ± 0.13
Free Carnitine μmoles/ℓ	19.6 ± 4.9	22.5 ± 3
Total Carnitine μmoles/ℓ	28.0 ± 2.2	47.7 ± 4.7[a]
Acylcarnitine μmoles/ℓ	12.5 ± 1.3	29 ± 2.9[a]
CAT[c] nmoles/mg min/prot	69 ± 4.4	40 ± 4.0[a]
CPT[c] nmoles/mg min/prot	25.3 ± 0.51	22.1 ± 0.51[a]
FAS[d] nmoles/mg min/prot	25 ± 0.9	6.1 ± 2[a]
PEPcK[d] nmoles/mg min/prot	52 ± 1.7	55 ± 2.2

a) $p<0.01$ for difference between obese and lean pups.

Mean ± S.E.

b) For 70-day-old group only. 6 mice in each group.

For all other parameters 12 mice per group.

c) Activity per mg mitochondrial protein.

d) Activity per mg supernatant (100,000 x g) protein.

Table II shows that in both brown and white adipose tissue fatty acid synthetase activity is higher in lean than obese mice. This finding was confirmed in three independent experiments (not shown). It is also apparent from Table II that PEPcK activity in brown adipose tissue is considerably higher in lean mice than in their obese litter mates, while there is no difference in enzyme activity between the two groups as far as white adipose tissue is concerned. It should be noted here that it is not easy to isolate BAT from obese mice. The area between the shoulder blades is fully covered with a large layer of white fat and the preparation of the brown adipose tissue is made difficult by the fact that it differs from white fat more by its harder consistency than, as is usual in lean animals, by its color. As can be seen from Table II the total weight of BAT in obese is twice that found in lean animals. This probably underestimates the actual size of brown fat, since we took care to discard any doubtful parts of the tissue.

TABLE II

Parameters for Brown and White Adipose Tissue of Lean and Obese Mice Aged 50 - 70 Days

	Obese (12)	Lean (12)
Brown fat weight[b]	127 ± 13	55.5 ± 5.1[a]
Brown fat CAT[c]	400 ± 12	340 ± 7[a]
Brown fat FAS[c]	19 ± 1	41 ± 5[a]
Brown fat PEPcK[c]	21.4 ± 1.9	80.3 ± 11.46[a]
White fat FAS	6 ± 0.51	15 ± 2[a]
White fat PEPcK	6.3 ± 1.6	6.3 ± 2.7

a) $p<0.01$. Figures in parentheses - number of animals used.

b) For 70-day-old group only.
For further explanations, see Table I.

c) (nmoles.mg min/prot).

DISCUSSION

Our results show that during the relatively early stages of obesity in ob/ob mice, the activity of FAS is elevated in liver only but is depressed in brown and white fat. A surprising finding was the very high FAS activity in brown fat of lean animals which suggests that the tissue was going through a period of involution similar to that described in the rat (13). This is also indicated by the high activity of PEPcK in brown fat of lean mice, since in this tissue the enzyme is thought to be necessary for α-glycerophosphate formation for triglyceride synthesis (14).

The blood level of acylcarnitine was lower in young obese mice than in their lean litter mates as was the ratio of acyl- to free carnitine. Our previous work indicated that low serum levels of acylcarnitine and a low ratio reflect a low rate of lipolysis and of ketone formation (5). However, we found no significant difference between the two groups in the serum β-hydroxybutyrate levels. This may be due to a greater rate of utilization of ketones in the lean group.

There was no difference in the free carnitine levels between the two groups so that levels of total carnitine were significantly lower in obese mice. Contrary to expectations, the activity of carnitine acetyltransferase was elevated in the young obese group, as was that of palmitoyl carnitine transferase. Usually, hepatic transferases are elevated when fatty acid oxidation is high (starvation, high fat diet) (14), i.e. under conditions when the blood levels of ketones, free fatty acids and carnitine are elevated. Exactly the opposite situation prevails in the young obese mouse. Lower levels of serum acylcarnitine accompany elevated activity of both carnitine transferases. It is quite possible that in the obese mouse both fatty acid oxidation and fatty acid synthesis are elevated in the liver, as suggested by others (12). Increased entry of fatty acids into the liver would lead to their increased entry into the mitochondria and the acetyl-CoA formed would then be transported out of the mitochondria again with the aid of carnitine acetyltransferase as acetylcarnitine, which could then be reutilized in peripheral tissues for fatty acid synthesis or further oxidized in the Krebs cycle after being reconverted to acetyl-CoA by CAT in those tissues. A role for CAT in this sequence of events was postulated some years ago by (15).

Finally, the relatively low activity of FAS in white fat of young obese mice deserves special mention. Others have reported elevated lipogenesis from glucose (16) and triglyceride formation from acetate or glucose (17) in obese mice. However, the age of the animals was never given and one may assume that they were older than 6 weeks. From that aspect it is of interest that in pigs FAS activity is no higher in obese than in lean animals even though the rate of glucose incorporation into fatty acids is significantly greater in obese animals (18). It may be noted that in weaned obese rats fatty acids are synthetised at a significantly slower rate than in lean animals (19).

REFERENCES

1. DAVIS, T.R.A. and MAYER, J. Imperfect homeothermia in hereditary obese-hyperglycemiac syndrome of mice. Amer. J. Physiol. 177: 222-226, 1954.

2. HIMMS-HAGEN, J. Obesity may be due to a malfunctioning ob brown fat. Can. Med. Assn. J., 121: 1361-1364, 1979.

3. FRITZ, I.B. Carnitine and its role in fatty acid metabolism. In: Advances in Lipid Research. R. Paoletti and D. Kritcheoski (eds). Academic Press, N.Y., 285-334, 1963.

4. HAHN, P. and SKALA, J. The role of carnitine in brown adipose tissue of suckling rats. Comp. Physiol. Biochem., 51B: 507-515, 1975.

5. SECCOMBE, D.W., HAHN, P. and NOVAK, M. The effect of diet and development of blood levels of free and esterified carnitine in the rat. Biochim. Biophys. Acta., 528: 483-489, 1978.

6. FROHLICH, J., SECCOMBE, D.W., HAHN, P., DODEK, P. and HYNIE, I. Effect of fasting on free and esterified carnitine levels in human serum and urine. Metabolism, 27: 555-561, 1978.

7. SECCOMBE, D.W. and HAHN, P. Carnitine acetyltransferase in developing mammals. Biol. Neonate, 38: 90-95, 1980.

8. HAHN, P. and SKALA, J. Carnitine transferases in human fetal tissues. Biol. Neonate, 22: 9-15, 1973.

9. VOLPE, J.J., LYLES, T.O., RONCARI, A.K. and VAGELOS, P.R. Fatty acid synthesis of developing brain and liver. J. Biol. Chem., 248: 2502-2513, 1973.

10. HOPGOOD, M.J., BALLARD, F.J., RESHEF, L. and HANSON, R.W. Synthesis and degradation of phosphoenolpyruvate carboxykinase in rat liver and adipose tissue. Biochem. J., 134: 445-448, 1973.

11. LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. and RANDALL, R.J. Protein measurements with the Folin phenol reagent. J. Biol. Chem., 193: 265-275, 1951.

12. BRAY, G.A. and YORK, D.A. Hypothalamic and Genetic Obesity in Experimental Animals: an Autonomic and Endocrine Hypothesis. Physiol. Rev., 59: 719-809, 1979.

13. SKALA, J.P., HAHN, P., NOVAK, E. and DRUMMOND, G.I. Changes in interscapular brown adipose tissue of the rat during perinatal and early postnatal development. Intl. J. Biochem., 3: 225-242, 1972.

14. HAHN, P. and KIRBY, L.T. The effect of catecholamines, glucagon and diet on enzyme activities in brown fat and liver of the rat. Can. J. Biochem., 52: 739-743, 1974.

15. BRESSLER, R. and KATZ, R.I. The role of carnitine in aceto-acetate production and fatty acid synthesis. In: Recent Research on Carnitine - Its Relation to Lipid Metabolism. G. Wolf (ed). The M.I.T. Press. Cambridge, MA 65-81, 1965.

16. CARNIE, J.A., SMITH, D.G. and MAVRIS-VAVATANNIS, M. Effects of insulin on lipolysis and lipogenesis in adipocytes from genetically obese (ob/ob) mice. Biochem. J., 184: 107-112, 1979.

17. JAMDAR, S.C., SHAPIRO, D. and FALLON, H.J. Triacylglycerol biosynthesis in the adipose tissue of the obese-hyperglycemiac mouse. Biochem. J., 158: 327-334, 1976.

18. MARTIN, R.J. and HERBEIN, J.H. A comparison of the enzyme levels and the in vitro utilization of various substrates for lipogenesis in pair-fed lean and obese pigs. Proc. Soc. Exp. Biol. Med., 151: 231-235, 1976.

19. LOUIS, S.L. and DAVIS, G. Fat cells lipogenesis from dietary obese rats fed high fat or high carbohydrate diet. I. glucose, pyruvate and acetate metabolism. Nutr. Rep. Intl., Vol. 20: (1), 91-100.

NUTRITION RESEARCH, Vol. 1, pp. 101-106, 1981
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SINGLE NUTRIENT DEFICIENCY AND CELL-MEDIATED IMMUNE RESPONSES. II. PYRIDOXINE[1].

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ABSTRACT

Weanling rats given pyridoxine-deficient diet and 4-deoxypyridoxine hydrochloride in water showed a significant reduction in the weights of the thymus and the spleen as well as in the number of lymphocytes in these organs compared with values in pair-fed controls and animals fed ad libitum. The number of 'background' plaque forming cells in the spleen was increased but the number of post-immunization antibody-forming cells was reduced. Dose response curve for phytohemagglutinin-induced lymphocyte stimulation was significantly lower than in controls. Serum thymic hormone activity was decreased. These immunologic changes may result in altered vulnerability to infection.

KEY WORDS: Pyridoxine, cell-mediated immunity, thymic hormone.

Vitamin B_6 is essential for the metabolism of amino acids, lipids, carbohydrates and nucleic acids. Pyridoxal-5-phosphate serves as the co-enzyme for several enzyme systems that are involved in the metabolism and interconversion of amino acids, such as decarboxylation, transamination, and desulfuration. It is a constituent of phosphorylases responsible for glycogen degradation. Furthermore, it promotes the synthesis of neurotransmitters such as serotinin and catecholamines from their respective substrates tryptophan and tyrosine. The critical role of pyridoxine in DNA synthesis and cell replication suggests that pyridoxine deficiency would significantly impair the function of tissues with rapid turnover. The immune system falls into this category. We have examined the effects of isolated deficiency of pyridoxine on cellular immunity.

MATERIALS AND METHODS

Groups of weanling Sprague-Dawley rats weighing 35-40 g were fed a pyridoxine-deficient diet (Tekland) for 3-4 weeks until growth plateau. In addition, for the next 2 weeks, these animals were given drinking water containing 0.1 mg of 4-deoxypyridoxine hydrochloride per ml. Control animals were pair-fed standard laboratory diet or allowed food ad libitum. The animals were

[1] Presented as part of the Borden Award Lecture, Nutrition Society of Canada, Vancouver, June 21, 1979.

housed in individual plastic cages in a room controlled for temperature (21°), humidity (40-50%) and light (alternating 12-hour periods of light and dark automatically).

Some rats were killed to determine the weight of the thymus and the spleen, the number of lymphoid cells, and to evaluate mitogen stimulation response. 1×10^6 lymphocytes separated by centrifugation on a Ficoll-Hypaque density gradient were cultured in triplicate in RPMI-1640 supplemented with 10% heat inactivated fetal calf serum and penicillin-streptomycin-fungi zone. Phytohemagglutinin (PHA) was used in concentrations ranging from 0.12 to 5.0 µg per well in microtiter plates. After incubation for 72 hours at 37°C in humidified air and 5% CO_2, 1 µCi of ^{3}H-thymidine was added and the plates incubated again for 12 hours. The cells were then harvested and radioactivity counted in Beckman Liquid Scintillation Counter. Results were expressed as stimulation index defined as the ratio between counts per minute of PHA-containing cultures divided by counts per minute of cultures without PHA.

Other groups of control and pyridoxine-deficient rats were immunized intraperitoneally with 2×10^8 sheep red blood cells (SRBC) and killed 5 days later to estimate the number of antibody hemolytic plaque forming cells (PFC) in the spleen employing Jerne's technique (1). 'Background' PFC were estimated in the spleens of nonimmunized animals.

Serum thymic hormone activity was quantitated by the method of Dardenne and Bach (2). The blood was allowed to clot at 4° temperature in plastic centrifuge tubes. The serum was separated in a refrigerated centrifuge and filtered immuniately. TF activity was assayed either the same day when the blood was collected or within a few days of storage at -20°C. Spleen cells were obtained from 8-12-week-old male C57Bl/6J mice that had been thymectomized 10-20 days before they were killed. The spleen cells were dissociated, washed twice in Hanks solution, resuspended in a concentration of 30×10^6 cells/ml and incubated at 37° with serial $\log_2$ dilutions of test sera in Hanks medium and azathioprine 10 µg/ml. After 80 minutes, 1% suspension of SRBC was added. SRBC had been kept in Alsever's solution for up to 3 weeks and before use were washed two times with saline and twice with Hanks solution. The cell suspension was centrifuged at 4° for 5 minutes and resuspended gently. Rosettes were observed in a hemocytometer. The highest dilution of test serum sample which was able to restore azathioprine sensitivity, i.e. induce 50% inhibition of SRBC rosette-forming cells was taken as the concentration of thymic hormone activity in the serum.

Results were analysed by Student's t test.

RESULTS AND DISCUSSION

Nutritional deficiency is an important modulator of immune responses. Protein-calorie malnutrition is associated with significant alterations in several components of host defense (3-5). Our recent data on the effect of single nutrient deficiency on cell-mediated immunity in laboratory animals have been reported elsewhere (6, 7). In the present study, we have demonstrated that pyridoxine deprivation in growing rats resulted in marked reductions in the weight and lymphocyte population of the thymus and the spleen, PFC response, mitogen-stimulated lymphocyte proliferation and serum thymic hormone activity.

Pyridoxine deficient animals showed retarded growth. The use of pair-fed controls eliminated the complicating effects of reduced total intake. The experiments showed that dietary lack of pyridoxine resulted in involution of

the thymus and the spleen (Table 1) with corresponding decrease in the number

TABLE 1

Organ Weights

Group	Number of Animals	Thymus Weight (mg)	Spleen Weight (mg)
Pyridoxine-deficient	7	151 ± 29[a]	314 ± 39
Pair-fed control	7	233 ± 25	369 ± 46
p		< 0.05	NS[b]

[a] Mean ± standard deviation

[b] Not significant

of mononuclear cells in these organs. The mean ± SEM lymphocyte count in the thymus and the spleen was 141 ± 29 millions and 168 ± 23 millions respectively in the deprived group compared with 201 ± 37 millions and 289 ± 44 millions respectively in the pair-fed animals. These differences are statistically significant ($p < 0.05$). PFC response was significantly lower in the group fed vitamin B_6-deficient diet (Table 2). Antibody formation in response to heterologous red cells requires an active collaboration between T and B lymphocytes

TABLE 2

Plaque Forming Cell Response

Group	Number of Rats	Direct PFC per spleen ($\times 10^{-3}$)	
		Background	5th day post-immunization
Pyridoxine-deficient	7	0.49 ± 0.12[a]	21.7 ± 6.9
Pair-fed Control	7	0.21 ± 0.07	49.1 ± 7.8
p		< 0.01	< 0.05

[a] Mean ± standard deviation

and macrophages. It is not clear whether pyridoxine deficiency predominantly

affects one cell population. Interestingly, the number of 'background' plaques was increased (Table 2). This may be the result of a selective loss of cells capable normally of suppressing spontaneous PFC. The functional significance of these plaques is not clear; however, their number is increased in NZB mice preceding the development of autoimmune disease, and in old animals.

Lymphocyte stimulation response using PHA was reduced in pyridoxine deprived rats. The differences between the vitamin B_6-deficient group and controls were significant at all doses of PHA (Table 3). Both T and B lymphocytes

TABLE 3

Mitogen Stimulation Response

Group	Number of Rats	PHA-Induced Stimulation Index		
		0.2 μg[a]	0.5 μg	2.0 μg
Pyridoxine-deficient	7	29.1 ± 14.6[b]	37.6 ± 18.9	36.1 ± 21.7
Pair-fed Control	7	81.3 ± 29.3	117.8 ± 31.6	101.3 ± 37.5
p		< 0.05	< 0.01	< 0.01

[a] Amount of phytohemagglutinin (PHA) in each well of microtiter plate

[b] Mean ± standard deviation

are stimulated by this mitogen, predominantly the former. This observation complements data indicating the deleterious effects of pyridoxine deficiency on delayed hypersensitivity responses (8), mixed lymphocyte reaction (9), and thymic epithelial cell function (10). Our observation of reduced serum thymic hormone activity in the experimental group (Fig. 1) suggests that pyridoxine

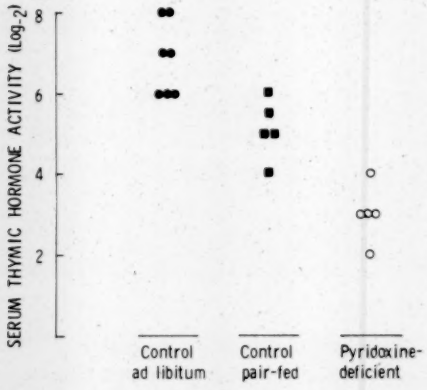

FIG. 1

Serum thymic hormone activity

is important for the normal production of thymic inductive factors (11).

The pathogenetic mechanisms of immunologic changes brought about by pyridoxine deficiency are probably multiple: reduction of DNA synthesis, impaired production of cellular proteins, prolonged cell division time, decreased synthesis or release of thymic indictive hormones (11), etc. A further possibility is the renewed synthesis of embryonic proteins with immunosuppressive properties. For example, a proportion of baboons fed vitamin B_6-deficient diet showed positive tests for α_1-fetoprotein in the serum (12). This protein has the ability to bind estrogens and possibly other substances and suppresses immune responses in vitro and in vivo (13). Nutritional deprivation during early periods of development results in increased α_1-fetoprotein levels (14).

Pyridoxine is an essential nutrient for numerous metabolic functions. Our studies reported here and those of other investigators (3-5) have demonstrated that dietary intake of pyridoxine is important for optimal cell-mediated immune responses.

REFERENCES

1. DRESSOR, D.W. and WORTIS, H.H. Use of an antiglobulin serum for detection of plaque forming cells with low haemolytic activity. Nature, 208: 859-861, 1965.

2. DARDENNE, M. and BACH, J.F. Studies on thymus products. II. Demonstration and characterization of thymic hormone. Immunology, 25: 353-366, 1973.

3. CHANDRA, R.K. and NEWBERNE, P.M. Nutrition, Immunity and Infection. Mechanisms of Interactions. Plenum, New York, 1977.

4. CHANDRA, R.K. Immunology of Nutritional Disorders. Arnold, London, 1980.

5. SUSKIND, R. Malnutrition and the Immune Response. Raven Press, New York 1977.

6. CHANDRA, R.K. and AU, B. Single nutrient deficiency and cell-mediated immune responses. I. Zinc. Am. J. Clin. Nutr. 33: 736-738, 1980.

7. CHANDRA, R.K. and AU, B. Single nutrient deficiency and cell-mediated immune responses. III. Vitamin A. Nutr. Res. (in press).

8. AKPOM, C.A. and WARREN, K.S. The inhibition of granuloma formation around Schistosoma mansoni eggs. VI. Protein, calorie and vitamin deficiency. Am. J. Path. 79: 435, 1975.

9. ROBSON, L.C. and SCHWARZ, M.R. Vitamin B_6 deficiency and the lymphoid system. Cell. Immunol. 16: 135-142, 1975.

10. WILLIS-CARR, J.I. and ST. PIERRE, R.L. Effects of vitamin B_6 deficiency on thymic epithelial cells and T lymphocyte differentiation. J. Immunol. 120: 1153-1159, 1978.

11. CHANDRA, R.K., HERESI, G. and AU, B. Serum thymic factor activity in deficiencies of calories, zinc, vitamin A and pyridoxine. Clin. Exp. Immunol. 42: 332-335, 1980.

12. FOY, H., KONDI, A., LINSELL, C.A., PARKER, A.M. and SIZARET, P. Positive α_1-foetoprotein tests in pyridoxine deprived baboons: Relevance to liver carcinoma in Africans. Nature, 225: 952-953, 1970.

13. CHANDRA, R.K. Functional significance of alpha-fetoprotein during pregnancy. Immunosuppression, estrogen binding and morphogenesis. In: Transmission of Proteins Across Living Membranes. W.A. Hemmings (ed) Elsevier-North Holland, Amsterdam, 1979, pp. 423-425.

14. CHANDRA, R.K. and BHUJWALA, R.A. Elevated alpha-fetoprotein and impaired immune response in malnutrition. Internat. Arch. Allergy Appl. Immunol. 53: 180-183, 1977.

NUTRITION RESEARCH, Vol. 1, pp. 107-115, 1981
0271-5317/81/010107-09$02.00/0 Printed in the USA.
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THE EFFECTS OF ZINC AND CALCIUM DEFICIENCIES ON THE RATE OF BONE RESORPTION IN THE RAT

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ABSTRACT

An effect of zinc deficiency on bone resorption was assessed in rats, by measuring the rate of resorption in rapidly growing animals and a pathologically increased resorption secondary to calcium deficiency. Bone resorption was quantitated by the loss of ^{3}H-tetracycline from previously labeled bone. There was no effect of zinc deficiency on bone resorption in either normally growing or calcium deficient animals. Calcium deficiency alleviated the effect of zinc deficiency on body weight. In calcium deficient animals, bone zinc concentration was almost double that of calcium supplemented animals, suggesting a partial substitution of zinc for calcium in bone mineral.

KEY WORDS: Zinc - calcium - bone resorption

INTRODUCTION

Zinc plays a fundamental role in bone metabolism. Bone formation is reduced during zinc deficiency, as manifested by a reduction in osteoblastic activity (1), collagen and chondroitin sulfate synthesis (2,3) and alkaline phosphatase (4). Skeletal abnormalities are a prominent feature in animals made zinc deficient during development, due at least in part to the lack of development of centers of ossification (5). Since both DNA and RNA polymerases are zinc metalloenzymes (6), the reduced bone formation in zinc deficiency may be largely due to a generalized reduction in cellular proliferation and protein synthesis rather than a specific effect on collagen synthesis or some other component of bone formation.

A possible role for zinc in bone resorption has not been thoroughly explored. Hurley et al (7), on the basis of ^{45}Ca kinetic data, reported that bone resorption was reduced in zinc deficient rats. Two zinc metalloenzymes, carbonic anhydrase (6) and collagenase (8), are considered to be important in bone resorption; while bone carbonic anhydrase was not influenced by zinc deficiency (4), collagenase activity was reduced by 40-80% (9). The

proliferation and differentiation of osteoclasts could also conceivably be reduced in zinc deficiency.

On the other hand, bone has one of the highest zinc concentrations of all tissues (10), and in rapidly growing animals part of this zinc is available for utilization during subsequent periods of zinc deficiency (11). The teratogenic effects of zinc deficiency can be alleviated by an enhanced rate of bone resorption, resulting in an increased availability of skeletal stores of zinc (12). These observations do not necessarily imply an increased rate of bone resorption in response to zinc deficiency. They do, however, suggest that bone resorption may be less severely influenced than other metabolic processes because zinc becomes available to cells at sites where resorption is occurring. Urinary cyclic AMP levels, considered to be a highly specific measure of circulating parathyroid hormone (13), are elevated in zinc deficiency (14), indicating an elevated stimulus to bone resorption.

We have attempted to determine the direction and extent of an effect of zinc deficiency on bone resorption by measuring directly the rate of bone resorption in rats. Klein and Jackman (15) have described a method for quantitating bone resorption based on the release of ^{3}H-tetracycline previously incorporated into bone. Tetracycline (TC) is initially deposited at sites of mineralization, but is not recycled and redeposited when resorption of a labeled site occurs. Thus the loss of TC during a given period represents the loss of previously labeled bone. With an appropriate labeling schedule completed before a dietary deficiency is imposed it is possible to assess the effect of that deficiency on the rate of bone resorption. We have applied the technique to the effect of zinc deficiency on bone resorption occurring during normal growth in weanling animals and on an accelerated rate of resorption produced by a simultaneous calcium deficiency.

METHODS

1. Diets: The zinc deficient diet of Luecke et al (16) was used as the basal diet, but with $CaCO_3$, dibasic calcium phosphate and TC omitted. The calcium content of the diet was adjusted by addition of $CaCO_3$ to yield a calcium deficient (0.02% Ca by analysis) or calcium supplemented (0.65% Ca) diet. The zinc supplemented diet contained 36-38 ppm Zn, added as $ZnCl_2$, compared with a zinc deficient diet of 0.5-0.9 ppm Zn.

During the experimental period, all animals were housed individually in hanging, stainless steel cages free of solder. Deionized water was provided *ad libitum*.

2. ^{3}H-Tetracycline labeling of bone: The protocol of Klein and Jackman (15) for labeling rats that are to be placed on experimental regimens at weaning, was used. Pregnant dams (Holtzman Co., Madison, Wisconsin) were given daily subcutaneous injections of 20 μCi ^{3}H-TC on days 14-21 of gestation, to ensure homogeneous labeling of the fetal skeleton during its maximal growth phase. Litters were reduced to 8 pups (4 male, 4 female) 7 days after birth, and weaned at 23 days. Dams were fed standard laboratory chow and tap water throughout lactation.

3. Experimental design: Since pups of both sexes were obtained via in utero labeling, two separate studies were designed to take advantage of all animals available.

a. The effect of zinc on the rate of bone resorption: Male litter-mate rat pups were assigned to one of three feeding schedules for 28 days: (1) zinc-deficient, ad libitum-fed, (2) zinc-sufficient, pair-fed, or (3) zinc-sufficient, ad libitum fed. The fourth litter-mate was killed at weaning as a time zero control. Ten litter-mate sets were assigned to the study but three were excluded through loss of one member of the set.

b. The effects of zinc and calcium on the rate of bone resorption: Female pups were killed at weaning (time zero control, 9 animals) or randomly assigned to one of four feeding schedules: (1) calcium-deficient, zinc-deficient; (2) calcium-deficient, zinc-sufficient; (3) calcium-sufficient, zinc-deficient; or (4) calcium-sufficient, zinc-sufficient. Animals were pair-fed against the group which consumed the least amount of food (zinc deficient, calcium supplemented), and killed on day 28 of feeding.

4. Analytical and statistical techniques: Both humeri were cleaned of adhering soft tissue and marrow. One humerus was ashed at 550°C overnight to determine ash weight. The calcium and zinc contents of the ashed humerus were determined by atomic absorption spectrophotometry. The remaining humerus was dissolved in 3 ml 6 N HCl, and the ^{3}H content of duplicate 0.5 ml aliquots of the digest was determined by liquid scintillation spectrometry. Total cpm ^{3}H-TC per humerus were calculated for all groups.

The extent of bone resorption during the 4 week experimental period (Δ ^{3}H-TC/humerus) was calculated by subtracting the total cpm/humerus at the end of the study from the total cpm/humerus in weanling animals. In the study using male rats it was possible to calculate the loss of ^{3}H-TC on a within-litter basis to minimize differences due to variations in the extent of in utero labeling, and groups were compared using Student's paired t-test. The study of combined zinc and calcium deficiencies required a time zero group plus four experimental groups, precluding within-litter comparisons. Individual values from the 4 week experimental groups were subtracted from the mean of the time zero group. In this study a 2x2 factorial analysis of data was conducted (17).

RESULTS

1. Influence of zinc deficiency on bone resorption: Zinc deficiency was confirmed in male rats by the reduced body weight and humerus size relative to both pair fed and ad libitum zinc supplemented groups, and by bone zinc levels (Table 1). The concentration of zinc in the humerus of the zinc deficient group was 177 ± 9 ppm, compared with more than 450 ppm in the zinc supplemented groups, and the total zinc per humerus in the zinc deficient group was only approximately 20-30% of that in the zinc supplemented groups.

TABLE 1

Body Weight and Humerus Data for Male Rats After 4 Weeks of Experimental Diets (all values mean ± S.E.M. for 7 animals per group).

	Zinc deficient	Zinc supplemented	
		Pair fed	Ad libitum fed
Body weight (g)	100±6*	114±2	224±8
Humerus			
Ash wt (mg)	43±3**	53±1	68±2
mg Ca/humerus	16.3±1.2*	19.7±0.5	25.2±0.7
ppm Zn	177±9***	458±15	490±21
mg Zn/humerus	7.6±0.6***	24.1±0.8	32.7±2.1

In all comparisons, zinc deficient group was significantly lower than zinc supplemented ad libitum fed group at the $p<0.001$ level, and significantly less than zinc supplemented pair-fed group at $p<0.05$(*), $p<0.01$(**) or $p<0.001$(***) levels (Student's paired t test).

The ^{3}H-TC content of the humeri at the beginning of the study averaged 1240 ± 77 cpm/humerus (mean ± S.E.M., n=10). Approximately 40-45% of the labeled bone was lost during the four week experimental period (Table 2). There was no effect of zinc intake on the loss of ^{3}H-TC, indicating that the rates of bone resorption were comparable in all groups.

TABLE 2

Influence of Zinc Deficiency on Bone Resorption as Measured by Loss of ^{3}H-tetracycline from Pre-labeled Humeri (mean ± S.E.M., n=6 or 7).

	Zinc deficient	Zinc supplemented	
		Pair fed	Ad libitum fed
Total ^{3}H-TC/humerus (cpm)[a]	710±40	665±23	645±62
Δ ^{3}H-TC/humerus (cpm)[b]	-516±73	-586±74	-570±104
% ^{3}H-TC lost/humerus	40.3±3.8	46.0±3.2	44.3±5.7

[a]Total cpm/humerus after 4 weeks on the experimental regimen

[b]Difference between total cpm/humerus at beginning and end of 4 week experimental period, based on litter-mate values.

2. Combined deficiencies of zinc and calcium: The effect of zinc deficiency alone in the absence of calcium deficiency resulted in the lowest body weight, although the combination of both zinc and calcium deficiency led to the lowest ash weight and calcium content of the humerus (Table 3). The zinc content and zinc concentration of the humerus in the zinc deficient groups were only 25-30% of values for the zinc supplemented groups. Calcium deficiency alone led to a very high humerus zinc concentration (952 ± 15 ppm vs 553 ± 14 ppm in calcium supplemented animals), although total bone zinc content was lower because of the effect of calcium deficiency on humerus ash weight. The analysis of variance is shown only for bone zinc concentrations, but similar results were obtained for all bone parameters. In contrast to the elevated bone zinc concentration in calcium deficient animals, the ash weight, mg calcium/humerus and µg zinc/humerus all showed significant reductions in response to both calcium and zinc deficiency.

TABLE 3

Influence of Zinc and Calcium Intakes on Body Weight and Humerus Data of Female Rats (mean ± S.E.M., n=9 for all groups).

	Calcium supplemented		Calcium deficient	
	Zinc suppl.	Zn deficient	Zn suppl.	Zn deficient
Body weight	111±2	98±5	111±1	110±2
Humerus				
Ash wt (mg)	55±3	47±2	21±1	20±4
mg Ca/humerus	20.6±1.0	17.5±0.6	7.7±0.3	7.2±0.1
ppm Zn	553±14	181±9	952±15	237±15
µg Zn/humerus	30.3±1.1	8.4±0.6	20.3±0.7	4.7±0.4

Analysis of variance for ppm Zn

Source of variation	d.f.	Mean square	F	p
Between Zn	1	2,661,901.01	1680.65	0.01
Between Ca	1	463,715.60	292.77	0.01
Interaction Zn x Ca	1	264,710.26	167.13	0.01
Error	32	1,583.85		

In calcium supplemented animals the ^{3}H-TC contents of the humerus were similar to those of male animals, and 40-50% of labeled bone was lost during the 4 week experimental period (Table 4). A simultaneous calcium deficiency resulted in a loss of more than 85% of labeled bone. In both calcium

supplemented and calcium deficient groups there was not a significant effect of zinc deficiency on the rate of bone resorption.

TABLE 4

Influence of Zinc and Calcium Intakes on Rate of Bone Resorption as Measured by ^{3}H-tetracycline Loss from the Humerus in Female Rats (mean ± S.E.M., n=9 for all groups).

	Calcium supplemented		Calcium deficient	
	Zinc supp.	Zn deficient	Zn suppl.	Zn deficient
Total ^{3}H-TC/ humerus[a]	665±40	622±53	161±13	140±19
Δ ^{3}H-TC/humerus[b]	-474±40	-556±59	-978±13	-999±19
% ^{3}H-TC lost	41.6±3.5	48.9±5.2	85.9±1.1	87.7±1.6

[a]Final cpm/humerus after 4 week experimental period
[b]Loss of ^{3}H-TC during 4 week experimental period

Analysis of variance for % ^{3}H-TC lost

Source of variation	d.f.	Mean square	F	p
Between Zn	1	187.873	1.926	n.s.
Between Ca	1	15,560.068	159.570	0.01
Interaction Zn x Ca	1	66.259	0.679	n.s.
Error	32	97.512		

DISCUSSION

The rates of bone resorption measured in this study amounted to 40-50% of labeled bone in normal growing animals and 85-90% in calcium deficient females. These rates are in a range that should allow the detection of either an increase or a decrease in bone loss in response to zinc deficiency. There was no evidence from this study that zinc deficiency influenced either the rate of resorption accompanying normal growth or a pathologically increased rate of resorption secondary to calcium deficiency. These results are thus at variance with the observations of Hurley et al (6) of a decreased rate of resorption in zinc deficient rats. The ^{45}Ca kinetic method used in the latter study has been increasingly questioned as a valid measure of bone resorption (18,19), and the technique of Klein and Jackman (15) used in the present study was developed specifically to overcome the difficulties in interpretation of short-term tracer kinetic methods. The absence of recycling of ^{3}H-TC has been

well established, and appears to serve as an accurate, direct marker of the loss of bone mineral.

The interval of 3 weeks between the labeling of bone with ^{3}H-TC and the beginning of the dietary study prevented the uniform labeling of all bone because of the bone deposition accompanying rapid growth during that time. Nonetheless, the labeled bone was clearly accessible to resorption in view of the high percentages of ^{3}H-TC lost during the experimental period. Starcher et al (9) described a reduced turnover of bone collagen in zinc deficient chicks. In that study, ^{3}H-proline was administered after the chicks had become zinc deficient, and the loss of labeled hydroxyproline was measured 1-10 days after injection of isotope. The differences between their study and ours may reflect differences in turnover of recently deposited bone vs older, more highly mineralized bone, or alternatively differences in the mechanisms of removal of mineral and matrix during bone resorption.

There was clearly an association between zinc and calcium metabolism. Calcium deficiency alleviated the severity of zinc deficiency, at least as measured by an improvement in body weight. This is consistent with observations of a reduced teratogenic effect of zinc deficiency by a simultaneous calcium deficiency in pregnant rats (5). It appears, however, that the increased availability of zinc is strictly secondary to the increased bone resorption rather than the zinc deficiency directly promoting bone resorption. An increase in urinary zinc in hyperparathyroidism (20) and a decrease in plasma zinc following parathyroid hormone administration (21) are both the opposite of calcium responses to parathyroid hormone, indicating major differences in homeostatic mechanisms for the two elements.

A markedly elevated bone zinc concentration in calcium deficient animals (950 ppm) relative to calcium supplemented animals (550 ppm) suggests that there may have been a partial substitution of zinc for calcium in bone mineral in response to the calcium deficiency. The extent of such a substitution, though resulting in a large increase in bone zinc concentration, would be extremely small in terms of the percentage of calcium replaced. Nevertheless, there was a highly significant interaction of the two elements under these conditions.

ACKNOWLEDGEMENTS

This study was supported by grants DE04475 and DE05242 from the National Institutes of Health, Bethesda, Maryland.

REFERENCES

1. BERGMAN, B., FRIBERG, U., LOHMANDER, S. and OBERG, T. The importance of zinc to cell proliferation in endochondral growth sites in the white rat. Scand. J. Dent. Res., 80:486-492, 1972.

2. MESSER, H.H. Influence of zinc deficiency on bone collagen synthesis in vitro. Fed. Proc., 38:559 (1979).

3. NIELSEN, F.H., DOWDY, R.P. and ZIPORIN, Z.Z. Effect of zinc deficiency on sulfur-35 and hexosamine metabolism in the epiphyseal plate and primary spongiosa of the chick. J. Nutr., 100: 903-907, 1970.

4. HUBER, A.M. and GERSHOFF, S.N. Effects of dietary zinc on the enzymes in the rat. J. Nutr., 103: 1175-1181, 1973.

5. HURLEY, L.S. and SWENERTON, H. Congenital malformations resulting from zinc deficiency in rats. Proc. Soc. Exp. Biol. Med., 123: 692-696, 1966.

6. RIORDAN, J.F. and VALLEE, B.L. Structure and function of zinc metalloenzymes. In: Trace Elements in Human Health and Disease. A.S. Prasad and D. Oberleas (eds.). Academic Press, New York, 1976, pp. 227-256.

7. HURLEY, L.S., GOWAN, J. and MILHAUD, G. Calcium metabolism in manganese-deficient and zinc-deficient rats. Proc. Soc. Exp. Biol. Med., 130: 856-860, 1969.

8. SELTZER, J.L., JEFFREY, J.J. and EISEN, A.Z. Evidence for mammalian collagenases as zinc ion metalloenzymes. Biochim. Biophys. Acta, 485: 179-187, 1977.

9. STARCHER, B.C., HILL, C.H. and MADARAS, J.G. Effect of zinc deficiency on bone collagenase and collagen turnover. J. Nutr., 110: 2095-2102, 1980.

10. BERGMAN, B. Concentration of zinc in some hard and soft tissues of rat determined by neutron activation analysis. Acta Radiol., 9: 420-432, 1970.

11. BROWN, E.D., CHAN, W. and SMITH, J.C. Bone mineralization during a developing zinc deficiency. Proc. Soc. Exp. Biol. Med., 157: 211-214, 1978.

12. HURLEY, L.S. and TAO, S-H. Alleviation of teratogenic effects of zinc deficiency by simultaneous lack of calcium. Am. J. Physiol., 222: 322-325, 1972.

13. BROADUS, A.E. Clinical cyclic nucleotide research. In: Advances in Cyclic Nucleotide Research. P. Greengard and G.A. Robison (eds.). Raven Press, New York, 1975, pp. 509-548.

14. MURRAY, E., SINGER, L., OPHAUG, R.H., and WONG, K.M. Effect of zinc deficiency on cyclic adenosine monophosphate levels in the rat. J. Dent. Res., 58A: 191, 1979.

15. KLEIN, L. and JACKMAN, K.V. Assay of bone resorption in vivo with ^{3}H-tetracycline. Calcif. Tiss. Res., 20: 275-290, 1976.

16. LUECKE, R.W., OLMAN, M.E. and BALTZER, B.V. Zinc deficiency in the rat: effect on serum and intestinal alkaline phosphatase activities. J. Nutr., 94: 344-350, 1968.

17. SNEDECOR, G.W. and COCHRAN, W.G. Statistical Methods, 6th Ed. Iowa State University Press, Ames, Iowa, 1967.

18. RASMUSSEN, H. and BORDIER, P. The Physiological and Cellular Basis of Metabolic Bone Disease. Williams and Wilkins, Baltimore, 1974, pp. 71-104.

19. KLEIN, L. Direct measurement of bone resorption and calcium conservation during vitamin D deficiency or hypervitaminosis D. Proc. Natl. Acad. Sci., 77: 1818-1822, 1980.

20. MALETTE, L. and HENKIN, R. Altered copper and zinc metabolism in primary hyperparathyroidism. Am. J. Med. Sci., 272: 167-172, 1976.

21. CHAUSMER, A.B., STEVENS, M.D. and ZEARS, R. Influence of parathyroid hormone and calcitonin on tissue zinc homeostasis in the rat. Metabolism, 29: 617-623, 1980.

NUTRITION RESEARCH, Vol. 1, pp. 117-137, 1981
0271-5317/81/010117-21$02.00/0 Printed in the USA.
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REVIEW ARTICLE

OBESITY IN INFANTS, CHILDREN, AND ADOLESCENTS IN THE UNITED STATES
I. IDENTIFICATION, NATURAL HISTORY, AND AFTEREFFECTS

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ABSTRACT

This review examines the indentification, natural history and after-effects of obesity in children and adolescents in the United States. Although the use of the triceps skinfold to establish the diagnosis of obesity is widely accepted, a precise relationship of excessive adiposity with morbidity has not yet been clearly established. Available epidemiologic studies suggest a uniform incidence of the disease throughout childhood and adolescence. The aftereffects of obesity include effects on growth, psychological dysfunction, orthopedic disorders, hypertension, and maturity onset diabetes mellitus. An important but neglected concept in the study of obesity is the potentially synergistic metabolic abnormaliites induced by the disease which may contribute to its persistence.

KEY WORDS: Obesity, Assessment, Epidemiology, Morbidity

"Malnutrition" is commonly employed as a term synonymous with protein-calorie undernutrition. In reality, malnutrition is a non-specific diagnosis equally appropriate to undernutrition, nutrient imbalance, or overnutrition.

As this review will show, the frequency and significance of obesity among infants, children, and adolescents in the United States rivals that of other forms of malnutrition in developing countries. Published reports indicate that 5% to 40% of children in this country are affected (1-5). The disorder is accompanied by significant psychological and physiological morbidity. As such, obesity constitutes a major public health problem. Energy intake in excess of expenditure is the mechanism by which obesity evolves, rather than the direct causative agent. Although no specific causes have been identified, the weight of evidence suggests its origins are multiple and environmental. Therapy is ineffective, whether directed at individuals or groups of obese children and adolescents. Finally, the success of preventive programs for infectious diseases and other forms of malnutrition in children suggests that prevention is the most cost-effective intervention for obesity of children and adolescents.

Obesity is currently a statistical rather than a pathological diagnosis. In the following section we discuss the measurement of body fat, and the rationale for use of triceps skinfold standards to identify obesity. We review the natural history of obesity as it is variously defined and consider the multiple aftereffects of excessive body fatness. The current lack of adequate diagnostic criteria relates closely to unsatisfactory evaluation of after-effects, which perforce limits difinitive conclusions regarding the natural

history.

IDENTIFICATION OF OBESITY

Obesity is determined by laboratory, clinical and field measurements. Laboratory methods provide the most exact quantification of body fat, although the cumbersome equipment and time required for most of these measurements precludes their practical application in clinical settings or field work. The value of anthropometric methods to determine obesity in the clinic or in the field depends on the strength of their correlations with laboratory methods.

Laboratory Measurement of Body Fat

Body density, potassium - 40 (^{40}K) counting, and isotope dilution are three widely used laboratory methods for determining body composition of children. Body density is determined by water displacement (6), corrected for residual air in the lungs. Since body weight and the densities of fat and lean body mass are known, the total quantity of fat can be computed.

The determination of lean body mass by ^{40}K counting relies on whole-body scintillation counting to record gamma emissions of the natural radioactive isotope of potassium ^{40}K (7). The relative constancy of the potassium content of lean body mass permits calculation of body composition; subtraction of lean body mass from total body weight yields body fat.

Total body water is determined by administering an isotope, such as deuterium or tritium oxide, and measuring its concentration in either plasma or urine after an equilibration period of several hours (8). Body fat is assumed to have a negligible water content. Since the water content of lean body mass is a known quantity, it is possible to approximate total body fat. The application and rationale for these three methods has been reviewed in detail (9,10).

Measurements of body fat in the same lean subjects by all three procedures yield correlation coefficients between measurements that range from $r = 0.60$ to $r = 0.86$ (11,12). Agreement between any two measurements is not consistently better than other possible combinations for lean subjects, indicating a comparable accuracy of all three methods.

In subjects with excessive body fat, ^{40}K counts are poorly reproduced (13,14). Moreover, the specialized equipment required for ^{40}K counting and body density determinations, and the ethical considerations involved in use of radioactive isotopes for children and adolescents, favor the use of stable isotopes to determine total body water in measurement of body composition at these ages.

Anthropometric Measurements of Body Fat

Since none of the three laboratory methods are readily applicable to clinical or field determinations, practical attention has focused on anthropometric procedures. Triceps skin thickness (TSF) and weight for height are the measures most widely used to assess body fat and its excess in children. Subscapular skinfold thickness is reliable, but occasionally objectionable because of the necessity to disrobe. Furthermore, the direct assessment of obesity by a measure of fatness such as the TSF (15,16) is more logical than that of weight for height, which may be confounded by other variables such as muscle mass or skeletal size.

Triceps skinfold is measured by skin calipers exerting a standard force at a site on the dorsum of the arm, midway between the acromion of the shoulder and olecranon process of the elbow. Weight for height expressed as a percentage (e.g., 120%) of ideal body weight (IBW) derives from a comparison of

actual weight with ideal body weight, using growth charts and controlling for height, age, and sex.

Correlations of triceps skinfolds with total body fat as determined by the three principal laboratory methods are shown in Table 1. In general, correlation coefficients are high, significant, and similar for all ages. Since the determination of body fat by ^{40}K counting requires that the child lie motionless for a half hour, and since body density is determined by submersion, these methods are impractical for children less than eight years old. Among older children, correlations of weight for height with total body fat, lean body mass, or total body water are generally lower than those for triceps skinfold thickness, and they have been examined less frequently. Among younger children, few data exist on correlations of any measure of total body fat with either triceps skinfolds or weight for height.

Table 1. Correlation Coefficients of Triceps Skinfold Thickness (TSF) and Weight For Height With Total Body Fat, Body Density, Total Body Water as Determined by Three Different Techniques

Author	Method	Group	Number Subjects	Variable	r
Forbes et al, 1961 (7)	Potassium-40	11-44 year old males	42	Total body fat and mean 6 skinfolds Total body fat and weight for height	0.80
Flynn et al, 1970 (17)		4-5 year old males and females	66	Total body fat and TSF	0.40
Forbes and Amirhakimi 1970 (18)		8-18 year old males 8-18 year old females		Total body fat and TSF Total body fat and TSF	0.72
Forbes, 1964 (19)		8-18 year old males and females	27	Total body fat and relative weight* Total body fat ant TSF	0.93 0.72
Pariskova, 1971 (20)	Body Density	9-12 year old males 13-16 year old males 9-12 year old females 13-16 year old females	66 57 56 62	Body density and TSF Body density and TSF Body density and TSF Body density and TSF	-0.85 -0.93 -0.74 -0.74
Seltzer et al, 1965 (15)		Obese 12-19 year old females	32	Body density and TSF	-0.69
Young et al, 1968 (21)		Premenarche 9-16 year old females		Body density and TSF	-0.76
		Postmenarche 9-16 year old females		Body density and TSF	-0.58
Heald et al, 1963 (22)		12-18 year old males	66	Body density and TSF	-0.87
Young et al, 1968 (23)	Total Body Water	9-16 year old females	104	Total body water and TSF	0.49
Brook, 1971 (24)		1-11 year old males and females	23	Lean Body mass and TSF	0.98

* Relative to 50th percentile for age.

Correlation of the triceps skinfold with total body fat is not good when body fat is markedly increased (19), due to impaired accuracy and reproducibility of fat fold measurements (W.H. Dietz, unpublished observations). In contrast, weight expressed as a percentage of the 50th percentile is linear throughout the range of total body fat in obese children and adolescents (19).

In summary, the use of the TSF to determine adiposity in populations of children or adolescents is empirically justified. It is logical to measure adiposity by a measure of fatness per se; among older children and adolescents, the TSF correlates well with total body fat determined by laboratory methods. Two important limitations restrict its general application: first, the correlation of skinfolds with total body fat has not been established for infants; second, the TSF is an indadequate measure of adiposity at higher levels of body fat.

Adipose Tissue Development in Children

Changes in body fat determined by TSF measurements in children past the age of one year (16) are in general agreement with determinations by isotope dilution (25,26), body density (20), and ^{40}K counting (27,28). Changes of fat by age and sex as measured by the TSF are shown in Figure 1 (16). The results differ significantly.

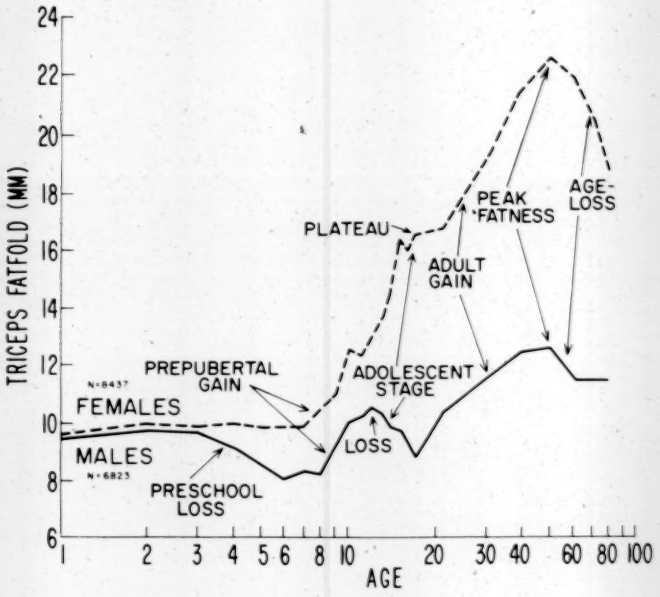

FIG. 1

Changes in fatness with age among males and females in the United States (reprinted with permission from Pediatrics (16), copyright American Academy of Pediatrics, 1976).

Males show a gradual increase in TSF between one and three years, followed by a loss between three and six years. The measurement then plateaus between six and nine years of age in males, followed by a prepubertal increase and an adolescent loss. Increases occur again in adulthood and continue until age 50 or 60 years. In females, the TSF thickness is essentially constant for the first eight or nine years. After that, adiposity tends to increase until after age 50, then declines.

Studies of adipocyte size and number in biopsy specimens fail to reflect sexual differences in development of adiposity (29-32). Adipocyte size increased to adult levels prior to one year of age, decreased between one and two years of age, and remained essentially constant thereafter until early adolescence, when cell size again increased to adult levels (32).

Small increases in cell numbers were observed from birth until age two years. From two until ten years, numbers remained constant, with a further increment after age 10. Increases in body fat are due primarily to an increase in cell size; the contribution of cell numbers is minimal (32).

Diagnosis of Obesity

The clinical diagnosis of obesity connotes a pathologic state. Since the accumulation of adipose tissue during states of excess energy intake is an adaptive physiologic response, the criteria for diagnosis of obesity should reflect the point at which this adaptive response has measurable pathologic consequences. Therefore, diagnosis depends not only upon reliable measurement of fatness, but also upon associated morbidity. Proof that obesity causes an accompanying disease requires (33): 1) that diverse approaches show strong, specific and consistent assoications; 2) a clear temporal relation of obesity to subsequent disease; 3) a dose effect, i.e., a rising risk of disease with increasing adiposity.

The aftereffects of obesity in children and adolescents have yet to be considered carefully from this perspective. For example, although a TSF in excess of the 85th percentile is a widely accepted diagnostic criterion for obesity (16,34), there is no *a priori* justification for its use to designate a pathologic state, except that statistically this group includes the fatter members of the population. Most studies have not examined the temporal relationship of obesity, defined by TSF, with other diseases of children, nor shown convincingly that the risk of other disease rises with the degree of adiposity. Despite these limitations, studies of several associated conditions partially support the use of a TSF greater than the 85th percentile to designate pathologic fatness.

Obese children often suffer severe peer group discrimination, suggesting that a practical diagnosis of obesity could be based on appearance. Interestingly enough, a highly significant correlation ($r = 0.64$ $p < .001$) (35) has been demonstrated between visual assessment of obesity and a triceps skinfold thickness beyond the 85th percentile.

Hypertension is an important disease of children and adolescents associated with excess adiposity (36,37). Although the correlation of systolic ($r = 0.39$) and diastolic ($r = 0.36$) blood pressures with TSF in a large population (36) was low, one Australian study (38) has shown a high correlation ($r = 0.90$, $r = 0.88$) of both systolic and diastolic pressures with triceps skinfolds roughly in excess of the 85th percentile.

Maturity onset diabetes mellitus is a common aftereffect of obesity in adulthood (39). Hyperinsulinemia, which may precede pancreatic exhaustion (4) has a large correlation with percent ideal body weight (41). Hyperinsulinemia also accompanies excess adiposity of children, and correlates with the degree of adiposity (42,43). Careful studies that relate hyperinsulinemia in children and adolescents to TSF have not been done; to the extent that TSF reflects adiposity, such a relationship would not be surprising.

Weight for height has been associated so infrequently with disease that its use as a diagnostic criterion for pathologic fatness cannot currently be justified.

Classification

Classification systems for obesity have been based on hypothetical etiologies (44-46), onanatomic distribution of fat (45), and on regulatory or metabolic causes (46). At present, no strong data support the utility of any one system to interpret morbidity in the individual or to promote understanding of the natural history of the condition in populations.

Since a diagnosis of obesity signifies a pathologic state, classification must start with a measurement of body fat. The TSF defines obesity but does not reflect its severity quantitatively (19). Weight expressed as a percentage of the 50th percentile for age correlates well with excessive body fat (19), but fails to reflect increments in height (47-50) or lean body mass (13,19) that often accompany excessive adiposity in both children and adolescents. Because no existing anthropometric measurements permit unequivocal

characterization of affected persons, the development of a classification system to predict morbidity and to clarify natural history awaits further research.

NATURAL HISTORY

Age at onset, and continuing studies of children who become obese, are crucial for prevention and control of obesity in either clinical or community settings. First, the correlation of age at onset with physiologic changes in adipose tissue mass, cell number, or cell size may suggest periods of high risk for development of persistence of obesity. Second, age at onset defines the particular nature and the relative size of populations at risk, thereby indicating the target as well as the likely methods of preventive or therapeutic intervention. Third, definition of the target population aids the development of hypotheses of cause. Finally, the true success of any intervention is assessed only by sound knowledge of the frequency and extent of spontaneous remission of obesity of varying severity. At each age, the information sought includes; a) the risk of the non-obese acquiring obesity; b) rates of spontaneous remission of obesity; c) the probability of persistent obesity; d) the correlation of severity of obesity with its duration.

Influence of Neonatal Adiposity

Pregnancy and the post-partum are times of high risk for both mothers and their infants to develop excess adiposity and its associated complications. Although no good prospective data establish the true incidence of obesity in women at this time, retrospective data suggest that 30% - 50% of persisting adult female obesity began during pregnancy or postpartum (51-53). Maternal obesity sometimes reflects serial transmission of the condition through successive generations, since significantly more obese mothers also had mothers who became obese during their pregnancies (54,55). Onset of obesity during pregnancy has been associated with greater frequency of stillbirths and neonatal deaths (54,55) as well as enhanced maternal morbidity during pregnancy and delivery (56).

Both pre-pregnant obesity and excessive weight gain of women during pregnancy have been associated with greater average birth weights (54-57) and increased adiposity (58,59) of their infants. The effect of excessive maternal weight or weight gain on infant adiposity remained, even when maternal populations were controlled for parity, smoking, blood pressure, and a family history of diabetes (58,59). However, abnormal glucose metabolism is frequent among the obese (30), and there is a positive correlation of maternal blood glucose levels and neonatal adiposity (60). Therefore, at least part of the effect of maternal obesity on neonatal adiposity might be attributable to its effect on maternal glucose metabolism.

The relationship of neonatal adiposity to subsequent obesity in infancy or childhood is more controversial. In a population of 500 infants of low income families in Philadelphia, weight for height at birth accounted for only 3% of observed variance in weight for height at four months of age (61). In contrast, another study (57) reported that 26% of newborns with a weight for height at birth in excess of the 95th percentile were still in that category when 4 years old. No significant correlation has been shown between skinfold thickness at birth and at one year of age (62).

OBESITY DEVELOPING IN INFANCY, CHILDHOOD, AND ADOLESCENCE

Incidence

Definitive studies of the incidence of obesity at any age in childhood or adolescence have not been done. Crude incidence rates can be deduced from studies where the same population is evaluated at different ages. Four such studies, each using different criteria to diagnose obesity, are shown in Table 2. Within the limits of these data, substantial differences of obesity at different ages are not apparent.

Table 2. Crude Incidence Rates (Cases per 1000 Children/Year) of Obesity in Males and Female Infants, Children, and Adolescents.

Author and Reference	Persons Studied	Sex	Age Period	Crude Incidence
Mack and Ipsen, 1974	1961	M*	4 months - 4 years	13.3
(61)	1687	F†	4 months - 4 years	16.0
Fisch et al, 1975	1345	M and F	birth - 4 years	12.5
(57)	1779	M and F	birth - 7 years	6.0
Hampton et al, 1966	447	M	14 - 18 years	21.3
(5)	505	F	14 - 18 years	19.8
Crisp et al, 1970	1603	M	7 - 15 years	12.7
(63)	1475	F	7 - 15 years	10.6
	1603	M	11 - 15 years	17.6
	1475	F	11 - 15 years	18.1

* Males

† Females

Relative Risk

Relative risk of a disease is calculated by comparing the prevalence of a disease in fractions of a population exposed and unexposed to a suspected factor. The relative risk of early obesity, defined by a variety of criteria, or rapid weight gain in infancy for development of later obesity, is presented in Table 3. All cited studies connect early obesity, absolute weight in infancy exceeding the 90th percentile, or a rapid weight gain at that time with a higher relative risk at later ages. In general, the risk increased with the age of the subjects when first examined. For example, a weight exceeding the 90th percentile in the first six months of life was associated with a twofold greater risk of obesity at 20-30 years of age (67), while obesity observed at 9-13 years of age had a sixteen-fold greater risk of continuing obesity 30-40 years later (68).

Case Control Studies and Cohort Studies

Case control (retrospective) and cohort (prospective) studies are the common epidemiologic methods used to determine age at onset and rates of remission, relapse, and return.

Case control studies compare the frequency of variables in affected and control populations. Table 4 summarizes a number of case control studies of age at onset of obesity.

Although diagnostic criteria varies widely, the results justify several tentative conclusions. Less than a third of children judged obese at age 4 or 5 years had been obese before one year of age. Summarizing the case control studies of 15 year old adolescents, approximately a third became obese prior to age 5 years, another third between 5 and 10 years of age, and the remainder between 10 and 15 years. Because no data represent the full period from birth

Table 3. Relative Risks of Selected Variables in Development of Obesity in Children and Adolescents

Author and Reference	Persons Studied	Variable Studied	Age at Follow-up	Relative Risk
Mack and Ipsen, 1974 (61)	1691 M*	relative weight < 1.5 S.D. from mean at 4 months	4 years	5.6
	1687 F†	relative weight < 1.5 S.D. from mean at 4 months	4 years	5.7
Mellbin and Vuille, 1973 (64)	465 M	weight gain greater than 7.5 Kg in infancy	7 years	2.4
		weight gain in any 3 months infancy < 97th percentile		8.1
		weight gain 1st and last 3 mo. infancy		19.3
	507 F	weight gain greater than 7.5 Kg in infancy	7 years	1.8
		weight gain in any 3 months infancy < 97th percentile		1.3
		weight gain in 1st and last 3 mo. infancy		1.3
Eid, 1970 (65)	214 M and F	rapid weight gain in 1st 6 months of infancy	6-8 years	4.75
Crisp et al, 1970 (63)	1603 M	> 1 S.D. from regression of weight on ponderal index at age 7	15 years	4.9
		> 1 S.D. from regression of weight on ponderal index at age 11	15 years	12.9
	1475 F	> 1 S.D. from regression of weight on ponderal index at age 7	15 years	7.4
		> 1 S.D. from regression of weight on ponderal index at age 11	15 years	16.0
Johnston and Mack, 1978 (66)	408 M	high relative weight compared to low relative weight	9-15 years	4.7
	390 F	at 1 year of age	9-15 years	5.9
Charney et al, 1976 (67)	366 M and F	infant weight > 90th percentile in 1st 6 mos. of age	20-30 years	2.0
Abraham et al, 1971 (68)	717 M	relative weight > 120% at age 9-13 years	39-53 years	15.9

*Males
†Females

to adolescence, the proportion contribution of obese infants to the adolescent obese cannot be specified. However, childhood and adolescent obesity apparently accounts for approximately a third of adult obesity (53,68). A further inference is that more girls than boys become obese during adolescence (4,71).

Table 5 illustrates the persistence of obesity in obese children after varying periods of observation. These data derive from studies of cohorts of obese children, again based on highly variable diagnostic criteria. In general, the probability of persisting obesity increased with age. For example, only 24% of male infants considered obese at 3 months were still obese when four years old (61). Thirty-one percent of seven year old obese males, and 54% of eleven year old obese males continued so at age 15 (63). Approximately 80% of obese sixteen year old adolescents were destined to become obese adults (75).

Followup studies of cohorts of obese children are also helpful to determine crude rates of spontaneous remission (Table 5). From one to four years of age, the rate appears to be about 25% per year. As expected from the data on persistence and relative risk, rates fall with advancing age; remission rates for obese persons between 7 and 15 years old averaged less than 10% per year. These observations are strengthened by a rising correlation of body somatotype with age through the age span of 4 to 20 years (63), and by a high correlation of skinfold thickness in children 6 to 11 years old with observations made six years later (76). These data contradict the popular notion that children and adolescents with "grow out" of obesity.

Only one study examined the effects of severity of obesity on remission rates (74). An experience with seven year old obese Swedish boys during a subse-quent eight year period indicated that remission occurred in approximately a half of those persons 2.00 to 2.49 standard deviations above the average weight/height ratio, but only in seven percent of those beyond 3.5 standard deviations.

Table 4. Age At Onset Of Obesity In Male and Female Children of Different Ages (in years)

Chief Author, Reference	Number of Obese Subjects	Birth	0.3	0.5	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	ADULT
Fisch et al, 1975 (57)	81 M* + F†	18% ‡					100%														
	91 M + F	18%						74%				100%									
Dine et al, 1979 (69)	48 M + F			40%		50%			100%												
Mack and Ipsen, 1974 (61)	111 M		34%		100%																
	113 F		44%		100%																
	111 M		23%				100%														
	113 F		25%				100%														
Charney et al, 1976 (67)	366 M + F			58%																	100%
Wilkinson et al, 1977 (68) (70)	67 M							25%						100%							
	94 F							54%						100%							
Asher, 1966 (71)	32 M							34%						72%				100%			
	69 F							20%						41%				100%			
Johnson, et al, 1956 (4)	M §							50%								80%					
								30%								60%		100%			
	F §																				
Crisp, et al, 1970 (63)	259 M										37%			56%				100%			
	236 F										47%			55%				100%			
Hampton, et al, 1966 (5)	89 M																93%			100%	
	93 F																86%			100%	
Mullins, 1958 (53)	101 M & F																		33%		100%

* Male
† Female
‡ Percentage of Obese Children
§ Number and precise ages not specified

Since adults with onset of obesity in childhood or adolescence arefatter than those with onset in adulthood (77), severity appears to be a crucial determinant of the behavior of the disease. Whether obesity of prolonged duration in childhood and adolescence also is associated with increased severity in adulthood is not clear; no studies among those reviewed examined this feature.

Unfortunately, no published data specify rates of relapse or return of obesity for any age.

Long term prospective epidemiologic studies of obesity in childhood and adolescence are relatively few; they usually pertain to different populations studied at different times, using widely divergent diagnostic criteria. These

limitations therefore support only a tentative conclusion that the incidence of obesity is constant throughout childhood and adolescence. The observations offer little support for an hypothesis that periods of adipocyte replication of rapid accumulations of body fat are periods of physiologic risk for the development of obesity.

AFTEREFFECTS

Obesity in children and adolescents is associated with both benign and morbid aftereffects (Table 6). A diagnosis of obesity describes a pathologic

Table 5. Persistence of Obesity in Obese Children of Different Ages (in years)

Chief Author, Reference	Number of Obese Subjects	Birth	0.3	0.5	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	ADULT
Fisch, et al, 1975 (57)	74 M* & F†	100%‡						26%			19%										
Mack and Ipsen 1974 (61)	106 M		100%		36%			24%													
	122 F		100%		41%			29%													
Huenemann, 1974 (72)	14 M & F			100%				25%													
Asher, 1966 (71)	32 M & F			100%				44%													
Charney, et al 1976 (67)	126 M & F				100%																36%
Sveger et al, 1975 (73)	18 M & F				100%	50%															
Johnson and Mack, 1978 (66)	20 M				100%											41% →					
	195 F				100%											32% →					
Crisp, et al, 1970 (63)	240 M										100%								31%		
	243 F										100%								46%		
	268 M														100%				54%		
	216 F														100%				60%		
Borjesson, 1962 (74)	164 M										100%								39%		
Abraham et al, 1971 (68)	19 M																100%				64%
Lloyd and Wolff, 1961 (75)	50 M & F																			100%	80%

* Males
† Females
‡ Obese Children

state. Therefore, excess adiposity, measured anthropometrically, should correlate with morbidity. In general, a precise relationship of excess adiposity to subsequent morbidity has yet to be established. Most studies have compared obese and non-obese children or adolescents without assessing the influence of other potentially important variables such as excess lean body mass, the distribution of adipose tissue, or determinable causes of excess adiposity.

Based on the limited information available, the term "aftereffects" appears more appropriate than "sequelae" to describe conditions associated with obesity; explicit proof (33) of a causal relationship has not emerged.

Table 6. Aftereffects of Obesity in Childhood and Adolescence

A. Changes in Growth and Development
B. Psychological Dysfunction
C. Respiratory Disorders
D. Orthopedic Conditions
E. Glucose Metabolism
F. Hypertension
G. Persistence into Adulthood

Growth and Maturation

Case control (78-79), prospective (81) and retrospective (49) cohort, and cross-sectional prevalence studies (47,82) all indicate that obesity in children and adolescents is associated with increased height. Gains in height appear much the same for boys and girls, averaging approximately 0.5 standard deviations above the mean expected for age and sex (82). One long-term study (49) indicated that the gain in height occurred coincidently with or followed the weight gain, suggesting that accretion of height is secondary to increased weight. No existing data indicate that the ultimate height achieved by obese children and adolescents is any greater than expected from parental heights.

Lean body mass also increases in obese children and adolescents (13,19) and may account for as much as 50% of the weight in excess of standard (13). This gain is apparently unrelated to the duration of obesity (13). Experimentally, excess energy intakes result in greater deposition of tissue protein (83), suggesting that chronic caloric intake in excess of expenditure can alone augment lean body mass. An alternative explanation is that greater weight-bearing produced muscle hypertrophy (13).

Bone age is advanced in about two-thirds of obese children and adolescents (79). Prospective observations have indicated that correlations of both height and bone age with adiposity are of the same order of magnitude (81). Since correlations of height age with bone age have not been sought, whether taller obese children and adolescents have advanced bone ages remains unclear.

Menarche occurs at earlier ages among obese girls than among the non-obese (79-81). Although adiposity as been proposed as an important determinant of menarche (84,85), this hypotheses has been challenged (86,87).

Psychological Dysfunction

Psychological dysfunction is an important aftereffect of childhood and adolescent obesity. Studies of six-year-old children show that they have already learned to associate stereotypes of personality and behavior with somatotype (88). In reference tests, normal children consistently ranked obese children below children with other handicaps (89,90). That obese adolescent girls are often passive, withdrawn, isolated, and concerned about their weight (91,92) is no surprise. These reactions resemble those arising from ethnic or racial prejudice (92). The obese thus constitute still another minority group subject to prejudice and descrimination (92).

Discrimination againstthe obese is not limited to peer group interactions.

College acceptance rates for obese adolescent girls are significantly less than for girls of normal weight (93). Physicians recognize obesity as a health problem in less than a half of severely overweight adults, and in only a fourth is any effort directed toward treatment (94). No comparable data are available for children or adolescents.

A variety of psychological studies comparing obese and non-obese preadolescents have failed to demonstrate differences in adjustment, happiness, self concept, or body image (95,96). However, using measures of socialization, substantial differences existed between obese and nonobese children, particularly in feelings of popularity or self-consciousness (W.H. Dietz, unpublished observations).

Adolescence appears to be the critical period for the formation of a negative self-image. Disturbances in body image of obese adults occurred almost exclusively among those who were overweight during childhood or adolescence (97), was most evident among persons who had been obese throughout childhood and adolescence, less among those who became obese during adolescence, and least among adults obese as children but not as adolescents (98).

In some families, obesity of the child or adolescent exerts an adaptive effect within family functioning (99), analogous to the role of a psychosomatic illness. The exact proportion of much cases among the obese has not been established, primarily because of methodolgic difficulties. Although parental overprotection and excessive dependency by obese children or adolescents is a frequent family characteristic (99), such reactions may represent a healthy supportive reaction to the handicap of obesity, rather than evidence of dysfunction. Prospective cohort studies of interactions among family members should clarify this problem.

Respiratory Disorders

Cohort studies of children less than two years old revealed an increased incidence of respiratory infections among children whose weight exceeded the 90th percentile (100) or whose weight for height was in excess of 120% of ideal (101).

A second respiratory effect of obesity in children and adolescents is the Pickwickian Syndrome. In contrast to the widely accepted pathogenetic model of obesity producing hypoventilation, pulmonary hypertension and right heart failure, it now appears that this disorder is a consequence of sleep apnea followed by repeated arousals (102). Lack of sound sleep, rather than hypercarbic narcosis, results in excessive daytime somnolence. Since the mortality of the Pickwickian syndrome in reported cases is 30%, effective therapy is urgent.

Orthopedic Conditions

Although slipped capital femoral epiphysis is rare among children and adolescents (103), approximately 30-90% of patients with this disorder are obese (104-106). Excessive weight presumably increases stress on the epiphyseal plate. Although sound data are lacking, prolonged inactivity during recovery may well contribute to further weight gain and, in turn, to later complications or recurrence of the hip disease.

Other orthopedic disorders in obese children and adolescents exhibit a similar relationship. We have seen children with Blount's disease, congenital hip dislocation, or severe bilateral genu varum, in whom obesity contributed to the orthopedic disorder.

Carbohydrate Metabolism

The most frequent abnormality among obese adults is basal hyperinsulinemia (107-109), a finding that correlates with both total body fat (109) and percent

ideal body weight (41). The insulin secretory response to glucose is enhanced in obesity (107,109). Over 65% of obese adults have impaired glucose tolerance (110) and more than a half of patients with adult diabetes are or have been obese (39). The basal hyperinsulinemia, augmented insulin secretory response to glucose, and impaired glucose tolerence often return to normal after weight reduction (107,111), implying that the changes are a consequence rather than a cause of obesity.

Abnormalities comparable to those of adults have been observed among obese children and adolescents. Hyperinsulinemia is common (42,43,112,113) and relates to the severity of obesity (42,113). An increased secretory response to glucose is freqent (43,113); abnormal glucose tolerance tests have been observed in 23-28% (112,113) of obese children and adolescnets. At least a moderate carbohydrate intolerance in children has been associated with a family history of diabetes (114,115). As with adults, many of the abnormalities are reversed by weight reduction (43).

Hypertension

Blood pressure determinations in obese children and adolescents suggests a common relation of obesity to hypertension. Among school children in Muscatine, Iowa (36,37) hypertension was more prevalent among the obese, although the data presented did not permit a precise comparison. The correlation of systolic and diastolic blood pressure was substantially greater for obese (38) than non-obese (36) children and adolescents (systolic pressure $r = 0.99$ vs. 0.39, diastolic pressure $r = 0.80$ vs. 0.36). More than a half of children with sustained hypertension were obese (37,116) and a fourth of obese children were hypertensive (38).

Persistence of Obesity into Adulthood

Evidence that the risk of persistent obesity rises with age was presented in the earlier discussion of natural history. Obesity in adults has been associated with job discrimination (117), hypertension (118-120), diabetes mellitus (40), and sudden death (121-123).

Synergy

The principle of synergy is a neglected feature in discussions of the aftereffects of obesity. Broadly defined, synergy is a "correlated action or cooperation" (124). The concept of synergy is well illustrated by the interaction of infection and nutrition in protein-calorie deficiency, where undernutrition increases susceptibility to infection and infection produces a further deterioration in nutritional state (125,126).

Potentially synergistic relationships also exist between the obesity of children and adolescents and a number of its aftereffects. Psychological dysfunction may produce bouts of excess eating, which further enhance adiposity and thereby add to low self esteem. Orthopedic aftereffects commonly reduce energy expenditure and increaseadiposity, with further deterioration of the orthopedic problem.

Obesity in childhood is accompanied by adipocyte hyperplasia. Since cell numbers cannot be reduced by weight loss (127), it has been suggested that adipocyte hyperplasia will maintain obesity (128,129). Although response to weight reduction therapy may depend on adipocyte cell numbers (130), careful comparisons of the effects of similar dietary restrictions on body composition in patients with hyperplastic or hypertrophic obesity have not been made.

Several metabolic aftereffects are potentially synergistic with obesity. Two regulatory enzymes for glycolysis and lipogenesis, phosphofructokinase and citrate cleavage enzyme, increase in adipose tissues of obese adolescents during hypocaloric dietary therapy (131), and may predispose obese patients to

regain weight after successful weight reduction. Likewise, the glycolytic and lipogenic roles of insulin, and the hyperinsulinemia accompanying excessive adiposity conceivably interact to perpetuate obesity.

Synergy represents an hitherto unexplored concept in obesity, but one which could unify diverse observations with the natural history of the disease. Likewise, since synergy expectedly perpetuates the disease, succesful therapy of obesity or of its aftereffects may depend on its resolution.

ACKNOWLEDGEMENTS

I wish to acknowledge the guidance and assistance of Dr. John E. Gordon in the development of this manuscript and the care of Ms. Priscilla Farry in its preparation.

REFERENCES

1. Center for Disease Control. Ten-state nutrition survey 1968-1970. Atlanta Center for Disease Control, 1972. (DHEW Publication No. [HSM] 72-8131).

2. STUNKARD, A., D'AQUILI, E., FOX, S., et al. Influence of social class on obesity and thinness in children. J. Am. Med. Assoc., 221:579-584, 1972.

3. RAUH, J.C., SCHUMSKY, D.A., and WITT, M.T. Heights, weights, and obesity in urban school children. Child. Dev., 28:515-530, 1967.

4. JOHNSON, M.L., BURKE, B.S., and MAYER, J. The prevalence and incidence of obesity in a cross-section of elementary and secondary school children. Am. J. Clin. Nutr., 4:231-238, 1956.

5. HAMPTON, M.C., HUENEMANN, R.L., SHAPIRO, L.R., et al. A longitudinal study of gross body composition and body conformation and their association with food and activity in a teen-age population. Am. J. Clin. Nutr., 19:422-435, 1966.

6. BEHNKE, A.R., Jr., FEEN, B.G., WELHAM, W.C. The specific gravity of healthy men. J. Am. Med. Assoc., 118:495-498, 1942.

7. FORBES, G.B., GALLUP, J., HURSH, J.B. Estimation of total body fat from potassium-40 content. Science, 133:101-102, 1961

8. SCHLOERB, P.R., FRIIS-HANSEN, B.J., EDELMAN, I.S., et al. The measurement of total body water in the human subject by deuterium oxide diluation. J. Clin. Invest., 29:1296-1310, 1950.

9. FORBES, G.B. Methods for determining composition of the human body. Pediatrics, 29:477-494, 1962.

10. BROZEK, J. Body composition. Science, 134:920-930, 1961.

11. CLARK, D.A., KAY, T.D., TATSEH. R.F., et al. Estimations of body composition by various methods. Aviat. Space. Environ. Med., 48:701-704, 1977.

12. KRYZWICKI, H.J., WARD, G.M., RAHMANN, R.A., et al. A comparison of methods for estimating human body composition. Am. J. Clin. Nutr. 27:1380-1385, 1974.

13. CHEEK, D.B., SCHULTZ, R.B., PARRA, A., et al. Overgrowth of lean and adipose tissues in adolescent obesity. Pediatr. Res., 4:268-279, 1970.

14. MERRITT, R.J. Therapy of pediatric obesity: effectiveness of current treatment and evaluation of protein supplemented fasting. Unpublished doctoral dissertation, Massachusetts Institute of Technology, 1977.

15. SELTZER, C.C., GOLDMAN, R.F., and MAYER, J. The triceps skinfold as a predictive measure of body density and body fat in obese adolescents girls. Pediatrics, 36:212-218, 1965.

16. GARN, S.M., and CLARK, D.C. Trends in fatness and the origins of obesity. Pediatrics, 57:443-456, 1976.

17. FLYNN, M.A., MURTHY, Y., CLARK, J., et al. Body composition of negro and white children. Arch. Environ. Health, 20:604-607, 1970.

18. FORBES, G., and AMIRHAKIMI, G.H. Skinfold thickness and body fat in children. Hum. Biol, 42:401-418, 1970.

19. FORBES, G.B. Lean body mass and fat in obese children. Pediatrics, 34: 308-314, 1964.

20. PARIZKOVA, J. Age trends in fat in normal and obese children. J. Appl. Physiol., 16:173-174, 1961.

21. YOUNG, C.M., SIPIN, S.S., and ROE, D.A. Body composition of pre-adolescent and adolescent girls. J. Am. Dietet. Assoc., 53:25-31, 1968.

22. HEALD, F.P., HUNT, E.E.,Jr., SCHWARTZ, R., et al. Measure of body fat and hydration in adolescent boys. Pediatrics, 31:226-239, 1963.

23. YOUNG, C.M., BOGAN, A.D., ROE, D.A., et al. Body composition of preadolescent and adolescent girls. J. Am. Dietet. Assoc., 53:579-587, 1968.

24. BROOK, C.G.D. Determination of body composition of children from skinfold measurements. Arch. Dis. Childh., 46:182-184, 1971.

25. FRIIS-HANSEN, B. Body water compartments in children: changes during growth and related changes in body composition. Pediatrics, 28:169-181, 1961.

26. EDELMAN , I.S., HALEY, H.B., SCHLOERB, P.R., et al. Further observations on total body water. Surg. Gynecol. Obstet. 95:1-12, 1952.

27. FORBES, G.B. and HURSH, J.B.: Age and sex trends in lean body mass calculated from K^{40} measurements: with a note on the theoretical basis for the procedure. Ann. N.Y. Acad., Sci., 110:255-263, 1963.

28. PIERSON , R.N., Jr., TIN, D.H.Y., and PHILLIPS, R.A.: Total-body potassium in health: effects of age, sex, height, and fat. Am. J. Physiol., 226:206-212, 1974.

29. HIRSCH, J. and KNITTLE, J.L. Cellularity of obese and nonobese human adipose tissue. Fed. Proc. 29:1516-1521.

30. HAGER, A., SJOSTROM, L., ARVIDSSON, B., et al. Body fat and adipose tissue cellularity in infants: a longitudinal study. Metabolism, 26:607-614, 1977.

31. BOULTON, T.J.C , DUNLOP, M. and COURT, J.M. The growth and development of fat cells in infancy. Pediatr. Res. 12:908-911, 1978.

32. KNITTLE, J.L., TIMMERS, K., and GINSBERG-FELLNER, F. The growth of adipose tissue in children and adolescents. J. Clin. Invest., 63:239-246, 1979.

33. United States Department of Health, Education and Welfare: Smoking and health: Support of the Advisory Committee to the Surgeon General. United States Government Printing Office, Washington, D.C., 1964.

34. SELTZER, C. and MAYER, J.: Simple criterion of obesity. Postgrad. Med., 38:A101-A107, 1965.

35. RAUH , J.L. and SCHUMSKY, D.A. Relative accuracy of visual assessment of juvenile obesity. J. Am. Dietet. Assoc., 55:461-464, 1969.

36. LAUER, R.M., CONNER, W.E., LEAVERTON, P.E. et al. Coronary heart disease risk factors in school children: the Muscatine study. J. Pediatr., 86: 697-706, 1975.

37. RAMES, L.K., CLARKE, W.R., CONNOR, W.E., et al. Normal blood pressures and the evaluation of sustained blood pressure elevation inchildhood: the Muscatine study. Pediatrics, 61:245-251, 1978.

38. COURT, J.M., HILL, G.J., DUNLOP, M. et al. Hypertension in childhood obesity. Aust. Pediatr. J., 10:296-300, 1974.

39. JOSLIN, E.P., DUBLIN, L.I., MARKS, H.H. Studies in diabetes mellitus IV. Etiology. Part 2. Am. J. Med. Sci., 192:9-23, 1936.

40. BERGER, M., MULLER, W.A., and RENOLD, A.E. Relationship of obesity to diabetes: some facts, many questions. In: Diabetes, Obesity, and Vascular Disease. H.W. Katzen and R.J. Mahler (ed.) Hemisphere Publishing Company, Washington, 1978, pp. 211-228.

41. BAGDADE, J.D., BIERMAN, E.L., and PORTE, D., Jr. The significance of basal insulin levels in the evaluation of the insulin response to glucose in diabetic and nondiabetic subjects. J. Clin. Invest., 46:1549-1557, 1967

42. PARRA, A., SCHULTZ, R.B., GRAYSTONE, J.E., et al. Correlative studies in obese children and adolescents concerning body composition and plasma insulin and growth hormone levels. Pediatr. Res., 5:606-613, 1971.

43. DESCHAMPS, I., DESJEUZ, J.F., MACHINOT, S., et al. Effects of diet and weight loss on plasma glucose, insulin, and free fatty acids in obese children. Pediatr. Res., 12:757-760, 1978.

44. BRAY, G.A. The Obese Patient. G.B. Saunders Company, Philadelphia, 1976.

45. BRAY, G.A. Definition, measurement and classification of the syndromes of obesity. Int. J. Ob., 2:99-112, 1978.

46. MAYER, J. Some aspects of the problem of regulation of food intake and obesity. N. Engl. J. Med., 274:662-672, 1966.

47. GARN, S.M. and CLARK, D.C. Nutrition, growth, development, and maturation: findings from the ten-state nutrition survey of 1968-1970. Pediatrics, 56:306-319, 1975.

48. WOLFF, O.H. Obesity in childhood. Quart. J. Med., 24:109-123, 1955.

49. FORBES, G.B. Nutrition and growth. J. Pediatr., 91:40-42, 1977.

50. RAUH, J.L. and SCHUMSKY, D.A. Lean and non-lean body mass estimates in urban school children. In: Human Growth. D.B. Cheek (ed.) Lea and Fabiger, Philadephia, 1968.

51. GURNEY, R. The hereditary factor in obesity. Arch. Int. Med., 57:557-561, 1936.

52. ANGEL, J.L. Constitution in female obesity. Am. J. Phys. Anthro., 7:433-471, 1949.

53. MULLINS, A.G. The prognosis in juvenile obesity. Arch. Dis. Childh, 33: 307-314, 1958.

54. RICHARDSON, J.S. The treatment of maternal obesity. Lancet, 1:525-528, 1952.

55. SHELDON, J.H. Maternal obesity. Lancet, 2:869-873, 1949.

56. EDWARDS , L.E., DICKES, W.F., ALTON, I.R., et al. Pregnancy in the massively obese: course, outcome and obesity prognosis of the infant. Am. J. Obstet. Gynecol., 131:479-483, 1978.

57. FISCH, R.O. BILEK, M.K., and ULSTROM, R. Obesity and leanness at birth and their relationship to body habitus in later childhood. Pediatrics, 56:521-527, 1975.

58. WHITELAW , A.G.L. Influence of maternal obesity on subcutaneous fat in the newborn. Brit. Med. J., 1:985-986, 1976.

59. UDALL , J.N., HARRISON, G.G., VAUCHER, Y., et al. Interaction of maternal and neonatal obesity. Pediatrics, 62:17-21, 1978.

60. WHITELAW, A. Subcutaneous fat in newborn infants of diabetic mothers: an indication of quality of diabetic control. Lancet, 1:15-18, 1977.

61. MACK, R.W. and IPSEN, J. The height-weight relationship in early childhood. Birth to 48 month correlations in an urban, low-income negro population. Hum. Biol., 46:21-32, 1974.

62. WHITELAW, A. Infant feeding and subcutaneous fat at birth and at one year. Lancet, 2:1098-1099, 1977.

63. CRISP, A.H., DOUGLAS, J.W.B., ROSS, J.M., et al. Some developmental aspects of disorders of weight. J. Psychosom. Res., 14:313-320, 1970.

64. MELLBIN, T. and VUILLE, J.C. Physical development at 7 years of age in relation to velocity of weight gain in infancy with special reference to incidence of overweight. Brit. J. Prev. Soc. Med., 27:225-235, 1973.

65. EID, E.E. Follow-up study of physical growth of children who had excessive weight gain in first six months of life. Brit. Med. J., 2: 74-76, 1970.

66. JOHNSTON, F.E., MACK, R.W. Obesity in urban black adolescents of high and low relative weight at 1 year of age. Am J. Dis. Child., 132:862-864, 1978.

67. CHARNEY, E., GOODMAN, H.C., MCBRIDGE, M., et al. Childhood atecedents of adult obesity. N. Engl. J. Med. 295:6-9, 1976

68. ABRAHAM , S., COLLINS, G., and NORDSIECK, M. Relationship of childhood weight status to morbidity in adults. HSMHA Health Reports, 86:273-284, 1971.

69. DINE, M.S., GARSIDE, P.S., GLUECK, C.J., et al. Where do the heaviest children come from? A prospective study of white children from birth to five years of age. Pediatrics, 63:1-7, 1979.

70. WILKINSON, P.W., PARKIN, J.M., PEARLSON, J., et al. Obesity in childhood: a community study in Newcastle upon Tyne. Lancet, 1:350-352, 1977.

71. ASHER, P. Fat babies and fat children. Arch. Dis. Childh., 41:672-673, 1966.

72. HEUNEMANN, R.L. Environmental factors associated with preschool obesity. J. Am. Dietet. Assoc., 64:488-491, 1974.

73. SVEGER, T., LINDBERG, T. WEIBULL, B., et al. Nutrition, overnutrition and obesity in the first year of life in Malmö, Sweden. Acta. Paediatr. Scand. 64:635-640, 1975.

74. BÖRJESON, M. Overweight children. Acta. Paediatr. 51:Suppl. 132, 1962.

75. LLOYD, J.K., WOLFF, O.H. and WHELAN, W.S.: Childhood obesity. Brit. Med. J., 2:145-148, 1961.

76. ZACK, P.M., HARLAN, W.R., LEAVERTON, P.E., et al. A longitudinal study of body fatness in childhood and adolescence. J. Pediatr., 95:126-130, 1979.

77. RIMM, I.J., and RIMM, A.A. Association between juvenile onset obesity and severe adult obesity in 73,532 women. Am. J. Pub. Health, 66:479-481, 1976.

78. BRUCH, H. Obesity in childhood I. Physical growth and development in obese children. Am. J. Dis. Child. 58:457-484, 1939.

79. BRUCH, H. Obesity in relation to puberty. J. Pediatr., 19:365-375, 1941.

80. LEMARQUAND, H.S. Comment. Proc. Roy. Soc. Med., 44:458-459, 1951.

81. GARN, S.M. and HASKELL, J.A. Fat thickness and developmental status in childhood and adolescence. Am. J. Dis. Child., 99:746-751, 1960.

82. GARN, S.M, CLARK, D.C. and GUIRE, K.E. Level of fatness and size attainment. Am. J. Phys. Anthrop. 40:447-450, 1974.

83. MUNRO, H.N. Carbohydrate and fat as factors in protein utilization and metabolism. Physiol. Rev., 31:449-488, 1951.

84. FRISCH, R.E. and REVELLE, R. Height and weight at menarche and a hypothesis of menarche. Arch. Dis. Childh., 46:695-701, 1971.

85. FRISCH, R.E. and REVELLE, R. Height and weight at menarche and a hypothesis of critical body weights and adolescent events. Science, 169: 397-399, 1970.

86. JOHNSTON, F.E., ROCHE, A.F., SCHELL, L.M., et al. Critical weight at menarche. Critique of a hypothesis. Am. J. Dis. Child., 129:19-23, 1975.

87. TRUSSELL, J. Menarche and fatness: re-examination of the critical body composition hypothesis. Science, 200:1506-1509, 1978.

88. STAFFIERI, J.R. A study of social stereotype of body image in children. J. Personal. Soc. Psych., 7:101-104, 1967.

89. GOODMAN, N., DORNBUSCH, S.M., RICHARDSON, S.A., et al. Variant reactions to physical disabilities. Am. Social. Rev., 28:429-434, 1963.

90. RICHARDSON, S.A., GOODMAN, N., HASDORF, A.H., et al. Cultural uniformity in reaction to physical disabilities. Am. Sociol. Rev., 26:241-247, 1961.

91. HAMMAR, S.L., CAMPBELL, M.M., CAMPBELL, V.A., et al. An interdisciplinary study of adolescent obesity. J. Pediatr., 80:373-383, 1972.

92. MONELLO, L.F., and MAYER, J. Obese adolescent girls: an unrecognized 'minority' group? Am. J. Clin. Nutr., 13:35-39, 1963.

93. CANNING, H., and MAYER, J. Obesity - its possible effect on college acceptance. N. Engl. J. Med., 275:1172-1174, 1966.

94. MADDOX, G.L., ANDERSON, C.F. and BOGDANOFF, M.D. Overweight as a problem of medical management in a public outpatient clinic. Am. J. Med. Sci., 252:394-402, 1966.

95. SALLADE, J. A comparison of the psychological adjustment of obese and non-obese children. J. Psychosom. Res., 17:89-96, 1973.

96. MURPHY , S. A study of obese children: Their anxiety level , self concept and body image. Unpublished master's thesis. Massachusetts Institute of Technology, 1977

97. STUNKARD, A., and M. MENDELSON. Obesity and the body image. I. Characteristics of disturbances in the body image of some obese persons. Am. J. Psychiat. 123:1296-1300, 1967.

98. STUNKARD, A., and V. BURT. Obesity and the body image II. Age of onset of disturbances in the body image. Am. J. Psychiat. 123:1443-1447, 1967.

99. BRUCH, H. Psychiatric aspects of obesity. Metabolism 6:461-465, 1967.

100. TRACEY, V.V., DE, N.C. and HARPER, J.R. Obesity and respiratory infection in infants and young children. Brit. Med. J. 1:16-18, 1971.

101. HUTCHINSON-SMITH, B.H. Obesity and respiratory infection in children. Brit. Med. J. 1:460-461, 1971.

102. SIMPSER, M.D., STRIEDER, D.J., WOHL,M.E., et al. Sleep apnea in a child with Pickwickian syndrome. Pediatrics, 60:290-293, 1977.

103. KELSEY J.L., KEGGI, K.J., and SOUTHWICK, W.O. The incidence and distribution of slipped capital femoral epiphysis in Connecticut and the Southeastern United States. J. Bone. Jt. Surg., 52A:1203-1216, 1970.

104. KELSEY, J.L., ACHESON, R.M., and KEGGI, K.J. The body build of patients with slipped capital femoral epiphysis. Am. J. Dis. Child., 124:276-281, 1972.

105. SORENSON, K.H. Slipped upper femoral epiphysis. Acta. Orthop. Scand., 39:499-517, 1968.

106. CHUNG, S. Diseases of the developing hip joint. Pediatr. Clin. N. Am. 24:857-870, 1977.

107. EL-KHODARY, A.Z., BALL, M.F., and OWEISS, I.M. Insulin secretion and body composition in obesity. Metabolism, 21:641-655, 1972.

108. PARLEY M., and KIPNIS, D.M. Plasma insulin repsonses to glucose and tolbutamide of normal weight and obese diabetic and nondiabetic subjects. Diabetes, 15:867-874, 1966.

109. KREISBERG, R.A., BOSHELL, B.R., DIPLACIDO, J., et al. Insulin secretion in obesity. N. Engl. J. Med., 276:314-319, 1967.

110. JOHN, H.H. A summary of the findings in 1100 glucose tolerance estimations. Endocrinol., 13:388-392, 1929.

111. OLEFSKY, J. REAVEN, G.M. and FARQUHAR, J.W. Effects of weight reduction on obesity. J. Clin. Invest., 53:64-76, 1974.

112. MARTIN, M.M. and MARTIN, A.L.A. Obesity, hyperinsulinism, and diabetes mellitus in childhood. J. Pediatr., 82:192-201, 1973.

113. DESCHAMPS, I., GIRON, B.J. and LASTRADET, H. Blood glucose, insulin, and free fatty acid levels during oral glucose tolerance tests in 158 obese children. Diabetes, 26:89-93, 1977.

114. PAULSEN, E.P., RICHENDORFER, L., and GINSBERG-FELLNER, F. Plasma glucose, free fatty acids, and immunoreactive insulin in sixty-six obese children. Diabetes, 17:261-269, 1968.

115. CHIUMELLO, G., GUERCIO, M.J., CARNELUTTI, M., et al. Relationship between obesity, chemical diabetes and beta pancreatic function in children. Diabetes, 18:238-243, 1969.

116. LONDE, S., BOURGOIGNIE, J.J., ROBSON, A.M., et al. Hypertension in apparently normal children. J. Pediatr., 78:569-577, 1971.

117. LAUDERBACK, L. Fat Power: Whatever You Weigh is Right. Hawthorne Books Inc., New York, 1970.

118. TYROLER , H.A., HEYDEN, S., and HAMES, C.G. Weight and hypertension: Evans County studies of blacks and whites. In: Epidemiology and Control of Hypertension. Paul, O., (ed.) Stratton Intercontinental Medical Book Corp., New York 1975, pp. 177-201.

119. KANNEL, W.B., BRAND, N., SKINNER, J.J., Jr., et al. The relation of adiposity to blood pressure and development of hypertension. Ann. Int. Med., 67:48-59, 1967.

120. CHIANG, B.N., PERLMAN, L.V., and EPSTEIN, F.H. Overweight and Hypertension. Circulation, 39:403-421, 1969.

121. SELTZER, C.C. Some re-evaluations of the build and blood pressure study, as related to ponderal index, somatotype, and mortality. N. Engl. J. Med. 274:254-259, 1966.

122. SELTZER, C.C. Overweight and obesity. Minn. Med., 52:1265-1274, 1969.

123. COMSTOCK, G.W., KENDRICK, M.A. and LIVESAY, V.T. Subcutaneous fatness and mortality. Am. J. Epidemiol., 83:548-563, 1966.

124. Dorland's Illustrated Medical Dictionary. Twenty-fourth edition. W.B. Saunders Company, New York, 1965.

125. MATA, L.J.: The Children of Santa Maria Cauque': MIT Press, Cambridge, 1978.

126. SCRIMSHAW, N.S., TAYLOR, C.E., GORDON, J.E. Interactions of Nutrition and Infection. World Health Organization Monograph Series No. 57, Geneva, 1968.

127. KNITTLE, J.L., and GINSBERG-FELLNER, F. Effect of weight reduction on in vitro adipose tissue lipolysis and cellularity in obese adolescents and adults. Diabetes, 21;754-761, 1972.

128. KNITTLE, J.L. Obesity in childhood: a problem in adipose tissue cellular development. J. Pediatr., 81:1048-1059, 1972

129. COATES, T.J. and THORESON, C.E. Treating obesity in children and adolescents: a review. Am. J. Public. Health, 68:143-151, 1978.

130. HÄGER, A., SJÖSTRÅM, L., ARVIDSSON, B., et al. Adipose tissue cellularity in obese school girls before and after dietary treatment. Am. J. Clin. Nutr., 31:68-75, 1978.

131. KATZ, D.P. The effect of diet on adipose tissue enzymes in obese pediatric patients. Doctoral thesis, Massachusetts Institute of Technology, 1979.

NUTRITION RESEARCH, Vol. 1, pp. 139-140, 1981
0271-5317/81/010139-02$02.00/0 Printed in the USA.
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REVIEWS OF BOOKS

Magnesium in Health and Disease

Marc A. Cantin and Mildred S. Seelig, Editors. SP Medical and Scientific Books, Jamaica, NY pp. 965, 1980. Price $200.00.

This voluminous publication includes the text of 102 papers presented at the 2nd International Symposium on Magnesium held in Montreal in mid-1976. It contains sections on methodology of magnesium determinations, intermediary metabolism, cellular membranes, immunity and allergy, hormones and vitamins, bone and cartilage, renal physiology and pathology, heart, blood vessels, neurophysiology and pathology, pregnancy and perinatology, clinical aspects and interrelationships with trace minerals and toxic substances. There is enormous variation in the quality of different contributions. The book is printed from camera-ready copy and the production values are fair. The lettering on some of the figures is barely legible. The delayed publication of the proceedings from four years after the symposium was held reduces the scientific value of the book. Discussion of papers is not included. Moreover, the prohibitive cost in these days of restricted budgets would detract its purchase even by institutional libraries.

Nutrition and Cancer

Jan vas Eys, Buford L. Nichols, Jr. and Mildred S. Seelig, Editors. SP Medical and Scientific Books, Jamaica, N.Y. pp. 297, 1979. Price $25.00.

This is a book of mixed virtues. The editors have made a valiant attempt at reviewing the relationship between nutrition and malignant disease. In recent years, the importance of dietary factors in the development of disease has been recognized. On the one hand, the quantity and quality of food can modulate host resistance and susceptibility to disease. Also, cancer often is associated with increased metabolic expenditure and loss resulting in undernutrition and even cachexia.

The first part of the book deals with diagnosis and pathogenesis of malnutrition in the hospitalized cancer patient, the effects of nutritional deficiency on immunity and on small bowel function and elements of nutritional therapy. The second part is a pot pourri of such varied topics as anorexia and learned food aversions, trace elements and cancer, and electrolyte regulation of medullary thyroid carcinoma.

The quality of material presented in various chapters varies from good to poor. Some topics are dealt with at considerable length, whereas others receive brief sketchy treatment. There is no unifying progression from basic science to clinical application. Some of the illustrations are substandard and the references cited date back to 1976 or earlier, although the book was published in 1979.

There is a need for an interpretative review of our current understanding of the interrelationship between nutrition and cancer. This book does not fulfill such a void.

Human Nutrition. 3A. Nutrition and the Adult

Roslyn B. Alfin-Slater and David Kritchevsky. Plenum, New York. pp. 290, 1980. Price $25.00.

This volume is a useful component of the series on Human Nutrition and deals with carbohydrates and fats as main suppliers of energy, protein and amino acids, and fibre. Most of the chapters are well written and give a balanced view of the subject. One would have liked to see more editorial patchwork since there is some overlap between the contents of various contributions. There are a few ambiguous and unsubstantiated statements, e.g. "The fat in the depots and liver of a child with kwashiorkor must have had its origin in dietary carbohydrate, ...". The production values are generally good but the lettering on some figures (e.g. figures 5 and 6 on pages 198-199) is barely legible. The contents of the series were "designed for the researcher or advanced student of nutritional sciences". The depth of the material in most chapters would make the book useful as initial reading before delving into original articles and more recent literature. Despite these few limitations, the text is a welcome addition to the growing literature on nutrition and should be acquired by most biomedical libraries.

Advances in Nutritional Research. Volume 2

Harold H. Draper, Editor. Plenum, New York. pp. 250, 1979. Price $27.50.

Ten review articles on various aspects of nutrition are brought together in this volume. The topics covered include regulation of energy metabolism in ruminants, influence of nutrition on metabolism of carcinogens, influence of nutritional status on susceptibility to infection, nutrition and osteoporosis, metabolism of hydrogen selenide and methylated selenides, microbial factors and neural lipids, atherosclerosis and nutrition, nutrition and colon cancer, and trace elements in carcinogenesis. The scientific content is of high quality throughout the volume and useful bibliography is appended to each chapter.

NUTRITION RESEARCH, Vol. 1, pp. 141-142, 1981
0271-5317/81/010141-02$02.00/0 Printed in the USA.
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CONFERENCE SUMMARY

Fourth Arnold O. Beckman Conference on Human Nutrition

The meeting organized by the American Association for Clinical Chemistry was held in picturesque Portsmouth, New Hampshire, October 20-22, 1980. Session I on Nutrition 1980 opened with ROBERT H. HERMAN discussing the theme of clinical nutrition as a medical speciality. He considered coronary heart disease, obesity, cirrhosis of the liver, some types of neoplasms and hypertension to be linked to dietary factors and suggested that appropriate dietary advice would be a cost-effective method of preventive medicine. GEORGE L. BLACKBURN discussed the clinical and biochemical nutritional assessment of the hospitalized patient. He mentioned the various types of protein-energy malnutrition encountered in adults and suggested that nutritional surveillance should be initiated by looking at body weight, serum albumin concentration and lymphocyte count. This can be supplemented by triceps skinfold thickness, mid-upper arm circumference, serum transferrin and delayed hypersensitivity skin tests. These data provide useful prognostic criteria. GEORGE M. OWEN summarized the results of recent nutritional surveys in the United States and emphasized that anemia, both iron deficiency and folate deficiency types, and obesity are the major problems. WILLIAM E. CONNOR analyzed the evidence for the nutritional prevention of several common disorders including the link between cholesterol and saturated fats and atherosclerosis, sucrose and dental caries, excessive calorie intake and disease, salt and hypertension, alcohol and liver disease, alcohol and atherosclerosis, carbohydrate and diabetes, and carbohydrate and atherosclerosis. ALFRED E. HARPER gave a skeptic's view of dietary goals for the United States. He stated the assumptions about relationships between diet and the incidence of chronic degenerative diseases that had been accepted as the basis for formulating food and nutrition policy and provided the scientific evidence for and against such assumptions.

Sessions II and III dealt with Nutrition and Specific Disease States. R.PHILIP EATON gave a detailed overview of lipoprotein metabolism. He discussed the macromolecular biochemistry and physiology of plasma lipoproteins, the kinetic model of apoprotein behaviour in man, hormonal modulation of cholesterol and other lipids, the population analysis of low- and high-density lipoproteins, the variations in their levels related to sex and region, and the clinical signi-ficance of hypercholesterolemia. PHILIP HOOPER discussed the role of dietary fat, carbohydrate, fibre and zinc upon the "anti-atherogenic" high-density lipoprotein. This topic was further elaborated by JAMES W. ANDERSON who provided data on the effect of high fibre diet with high carbohydrate on blood glucose levels of insulin-treated patients with diabetes mellitus. Unexpectedly, the fasting serum triglyceride values decreased. The glucose levels were lower and required smaller doses of insulin per day. Serum cholesterol levels fell significantly. He suggested that most adults with diabetes should be given a diet containing 50-60 % carbohydrate, 12-20 % fat, less than 300 mg cholesterol and at least 40 grams of plant fibre. RANJIT K. CHANDRA summarized the data on immunologic changes in under- and over-nutrition. Nutritional deficiency results in impaired delayed cutaneous hypersensitivity responses, lower numbers of circulating T lymphocytes, reduced serum thymic hormone activity, production of low affinity antibody, reduction in opsonization and intracellular killing of bacteria and fungi by phagocytes, and significant alterations in the complement

system. Deficiencies of single nutrients also result in discernible changes in immune responses. Obesity also is associated with a higher frequency of infections and altered immunocompetence, both in man and mice. These immunologic changes are likely to be linked to metabolic, hormonal and nutritional perturbations observed in overnutrition. CHARLES S. LIEBER talked on alcoholand liver disease. The generation of hydrogen can in part explain alterations in lipid and carbohydrate metabolism, collagen synthesis and impaired protein synthesis. Increased production of acetaldehyde is associated with impaired microtubule formation and function, protein retention and hepatocytic swelling. Acetaldehyde also interferes with mitochondrial oxidation and promotes peroxidation of cell membranes. What eventually attracts fibroblasts and promotes fibrosis is not established. Decreased protein intake in association with vitamin and other nutrient deficiencies may potentiate the direct toxicity of alcohol. DAVID H. LAW discussed the current view on the pathogenesis, causes and management of hepatic encephalopathy. The important role of elevated ammonia levels and deranged aminoacid concentrations was brought out. PAUL FERNHOFF reviewed various vitamin dependent diseases. The underlying mechanisms of vitamin responsivity include impaired transport of the nutrient into the cell, impaired conver-sion into the biologically active coenzyme, and impaired binding of the coenzyme with its apoenzyme. RUDOLPH LIEBEL gave an overview of the current concepts in pathophysiology of obesity. Changes in resting metabolic rate, the ubiquitous sodium-potassium ion pump and the mitochondrial cytochrome system, were discussed. Examples were cited of thermogenesis observed in many other animal species. He suggested that subtle biochemical explanations may underlie one's ability to maintain constant weight or to gain weight largely as a result of deposition of fat. DAPHNE ROE reviewed the extensive subject of interactions between drugs and nutrients and discussed the merits of different experimental animal models for investigation of such interactions.

Nutrition and Neurological Function was the theme of Session IV. BRIAN L.G. MORGAN summarized the data on the effects of nutritional deficiency on brain development, in particular the reversibility of cytological, biochemical and behaviour changes by appropriate environmental stimulation and by the administration of N-acetylneuraminic acid. ERNESTO POLITT provided evidence for the link between iron deficiency and learning and behaviour in preschool child-ren. The sideropenic children took more trials to reach a learning criterion in three discriminating learning tasks and made more errors in simple memory tasks. JOHN D. FERNSTROM discussed the biochemistry and regulation of brain neurotransmitter sysnthesis and release in response to dietary alterations. Protein free meal results in elevation of brain tryptophan and serotinin. He mentioned the effects on brain function of administering the precursors of neurotransmitters and the potential of these findings in the management of such disorders as tardive dyskinesia, depression and memory loss. The final session on Parenteral and Enteral Nutrition included a history and overview of parenteral nutrition by ARVID WRETLIND and practical consideration in feeding the hospitalized patient by BRUCE R. BISTRIAN. A selective review of trace elements essential for man was provided by HAROLD H. SANDSTEAD. The requirements of zinc, copper, chromium and other microminerals, their physiologic role, deficiency syndromes and therapy was discussed. HARRY L. GREENE dealt with Type I glycogenosis and PHILIP A. WALRAVENS summarized the laboratory monitoring of total parenteral therapy.

The proceedings of the Conference will be published in Clinical Chemistry in early 1981.

NUTRITION RESEARCH, Vol. 1, pp. 143-145, 1981
0271-5317/81/010143-03$02.00/0 Printed in the USA.
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ANNOUNCEMENTS

INTERNATIONAL CONGRESS OF NUTRITION

The XIIth International Congress of Nutrition is scheduled for August 16-21, 1981 in San Diego, California. The theme is Nutrition: Basic to Human Health and International Development.

The Congress is sponsored by the International Union of Nutritional Sciences, and is hosted in the United States by the American Institute of Nutrition, The American Society for Clinical Nutrition, and the National Academy of Sciences-National Research Council.

The symposia topics (and chairmen) are: Trace elements in human nutrition (A.S. Prasad), Nutrition and chronic disease (T.A. Tashev), Fat soluble vitamins (G. Brubacher), Nutritional status and susceptibility to disease (R. K. Chandra), Drug-nutrient interactions (A.E.M. McLean), Nutritional intervention programs (Luis F. Fajardo), Nutritional anemias (Leif Hallberg), Function of the gastrointestinal tract in health and disease (R. Braude), Nutrition component of national policy and planning (Fred T. Sai), Nutrition, brain function and behaviour (Josef Brozek), Marginal malnutrition: its assessment and functional consequences (Moises Behar), Protein-energy requirements and interactions (Benjamin Torun), Maternal, fetal and neonatal nutrition, (Priyani Soysa), and nutritional care of hospitalized patients (David Picou).

Mini-symposia include: Energy regulation in animals and man (E.M. Widdowson), Nutritional anthropology (Gretel Pelto), Nutritional epidemiology (R. Florentino), Water-soluble vitamins (V.A. Shaternikov), Protein-calorie malnutrition (Vinodini Reddy), Non-nutrient dietary components including fiber (David A.T. Southgate), Changing food patterns (Johanna Dwyer), Bioenergetics and nutrition of fish (Takeshi Nose), Nutrition and aging (Hamish Munro), Interactions between food components and their nutrition significance (James R. Kirk), Energy cost of food and nutrition systems (John Howthorne), Role of ruminants in producing nutrients for man (Werner Kaufman) and Novel sources of protein in animal and human nutrition (T.G. Taylor). Free Communications, presented orally or in poster sessions will be accepted in the following areas. The deadline for receipt of abstracts of free communications is February 28, 1981.

The registration fee is $150 if paid before July 1, 1981. Thereafter, registration will be $200. Payment of the fee entitles registrants entrance to all scientific sessions and exhibits, participation in the opening ceremonies, the first evening reception, a social event with dinner, and one copy of the final program with abstracts. Spouse registration fee will be $75, and entitles registered spouses to participate in all social events.

For registration materials and additional Congress information, contact: Secretariat, XII International Congress of Nutrition, 1629 K Street, NW, Suite #700, Washington, D.C. 20006, U.S.A.

SYMPOSIUM ON TRACE ELEMENTS

The Fourth International Symposium on Trace Element Metabolism in Man and Animals will be held in Perth, Australia, on May 11-15, 1981. The topics for various sessions include trace elements and development, absorption, storage and excretion, trace element deficiencies, toxicities and interactions, biochemical functions, immune responses and metabolism.

Correspondence about the Symposium should be addredded to Professor J. McC. Howell, School of Veterinary Studies, Murdoch University, Murdoch 6150, Western Australia.

SYMPOSIUM ON HUMAN MILK

The symposium will take place in Hradec Kralove, Czechoslovakia, May 18 to 21, 1981. It is organized by the Tissue Bank, Teaching Hospital, Hradec Kralove, under the auspices of C-1 Commission of the International Institute of Refrigeration, Paris, and the Low Temperature Biology Section of the Czechoslovak Academy of Sciences. For further information write to the Secretary of the Symposium, Ing. Lubomir Vavra, Fakultni nemocnice Kunz, Tkanova ustredna, 500 36 Hradec Kralove, Czechoslovakia.

MEDICAL STUDENT AWARD FOR RESEARCH IN CLINICAL NUTRITION

The purpose of the Medical Student Research Award program of the American Society for Clinical Nutrition (ASCN) is to encourage excellence in the research papers presented by medical students, and stimulate interest in clinical nutrition in medical schools.

Full-time medical students submitting abstracts to The American Society for Clinical Nutrition for its scientific program at the Annual Meeting are eligible to participate in the program. The student must be senior (first) author of the abstract, and must have played a major role in the planning and conduct of the research to be reported. Nominees must be sponsored by an ASCN member, and the sponsor must write a letter addressed to the Executive Officer of the ASCN affirming that the nominee is a full-time medical student and degree candidate specializing in some aspect of clinical nutrition. Further the sponsor must describe the nominee's role with respect to the research to be reported. The sponsoring letter (six copies) must accompany the abstract when it is mailed to the ASCN office, and six additional copies of the abstract must be submitted for use by the selection committee.

The recipient will receive a travel award to attend the ASCN Annual Meeting at which the paper is to be presented.

For additional information, contact the American Society for Clinical Nutrition, 9650 Rockville Pike, Bethesda, MD 20014.

AMERICAN BOARD OF NUTRITION CERTIFYING EXAMINATIONS

The American Board of Nutrition invites inquiries and applications from individuals desiring to be certified as Specialist in either Clinical Nutri-

tion or in the Human Nutrition Sciences.

The 1981 examinations will be given but two times. The next examination is scheduled for Thursday, April 23, 1981 in San Francisco, the day before the annual meeting of the American Society for Clinical Nutrition. The second examination in 1981 will be given on Saturday, August 15, 1981, in San Diego, the day before the XIIth International Congress of Nutrition.

For certification as Specialist in Clinical Nutrition, the individual must have earned an M.D. or equivalent degree, be certified by or be eligible for certification by one of the American Speciality Boards and have a minimum of two additional years of approved training and experience in the practice of clinical nutrition.

For certification as Specialist in the Human Nutrition Sciences, the candidate must have a doctorate degree, Ph.D., ScD., or M.D., or its equivalent, and a minimum of four post-doctoral years of approved training and experience in human nutrition.

Address inquires to the American Board of Nutrition, 9650 Rockville Pike, Bethesda, MD 20014.

NATIONAL RESEARCH SERVICE AWARDS IN NUTRITION

The National Institutes of Health announce the continuing availability of support for research training in nutrition. Domestic nonprofit, private, or public institutions may apply for grants to support training programs in nutrition research. Predoctoral and postdoctoral trainees may be supported. The training program director at the institution will be responsible for selecting trainees and overseeing the program. Applicants representing interested institutions are advised to contact persons designated in the various Institutes to discuss any questions before applying formally, especially if an application including predoctoral training is planned or if compatibility between institutional and agency training aims is in doubt. Names of these persons, other information, and application kits are available from the Office of Grants Inquiries, Division of Research Grants, National Institutes of Health, Room 448, Westwood Building, Bethesda, MD 20205.

FELLOWSHIP PROGRAM IN CLINICAL NUTRITION

This program is organized for pediatricians, internists, and surgeons who plan an academic career in medicine. The basic science aspects of the program will be provided at the Massachusetts Institute of Technology, and the clinical experience will be at the Children's Hospital Medical Center, the New England Deaconess Hospital, and the Boston University Hospital. Candidates must have an M.D. and two years of specialty training in pediatrics, medicine, or surgery. The M.S. or Ph.D. can be obtained. Send requests for information to Dr. Nevin S. Scrimshaw, Institute Professor, Department of Nutrition and Food Science, Massachusetts Institute of Technology, Room E17-438, Cambridge, MA 02142.

NUTRITION RESEARCH, Vol. 1, pg. 147, 1981
0271-5317/81/020147-01$02.00/0 Printed in the USA.
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EDITORIAL

Nutrition in Adolescence

None of the biomedical problems of adolescents are unique to that age group. This applies equally to nutrition. However, the sudden marked growth spurt, the dramatic changes in secondary sex features, the dynamics of adolescent behavior and psychosocial influences are characteristic and unparalleled. Growth at this period of life is accompanied by marked changes in body composition. There is an increase of lean body mass, mainly limb muscle and mineral skeleton. Signifi -cant alterations are noted in biochemical indices of nutrition. Thus there is an urgent need of obtaining normative data based on biological maturity rather than chronologic age. These changes in body constituents occur in both boys and girls. The age at which the rapid alterations occur varies greatly from one child to another, the transformation being earlier in girls. Moreover,girls are more generously endowed with adipose tissue than are boys.

There is surprisingly little actual data on the nutritional needs of adolescents. The current recommendations are based mainly on educated guesses and/or deductive judgment based on epidemiologic information, nutrient intake data and observations made in other age groups. Direct determinations by balance studies and body composition estimates are available only for a minority of nutrients for which there are recommended allowances. Furthermore, recommendations given by various expert groups vary enromously even though the estimates of requirements on which such advice is proferred vary much less. It has been suggested that increases in dietary intake during adolescence may be calculated by the average daily increments in body content of each nutrient from age 11-20 years and daily increments at the peak ofthe growth spurt. The rapid growth spurt du -ring adolescence demands increased intake of protein, iron, calcium, and zinc, as well as energy. Inadequate intake may delay or halt the pubertal process.

Food intake is governed by physiologic needs as well as psychosocial factors. The latter influences are particularly important during adolescence. The increa -sing opportunity to eat with peers away from the family unit, missing regular meals and eating at varying intervals, snacking, intake of alcohol and of soft drinks, are some of the dietary factors which may result in deficits or excesses of total nutrient intake or consumption of individual nutrients.Both under- and over- nutrition as well as imbalance may result in significant morbidity, both physical and emotional.

The important issue of nutrition in adolescence requires considerably greater attention than it has received so far.

R.K. Chandra

1. McKIGNEY, J.E. and MUNRO, H.N. (Editors) Nutrient Requirements in Adolescence. MIT Press, Cambridge, 1976.

2. EIGTH ANNUAL MARABOU SYMPOSIUM. Nutrition in Adolescence. Nutr.Rev. 39: 37-116, 1981.

NUTRITION RESEARCH, Vol. 1, pp. 149-157, 1981
0271-5317/81/020149-09$02.00/0 Printed in the USA.
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EFFECT OF TOTAL PARENTERAL NUTRITION IN THE RESTITUTION OF BODY NITROGEN, POTASSIUM AND WEIGHT

John R. Mernagh, Kenneth G. McNeill, Joan E. Harrison, Khursheed N. Jeejeebhoy.
Departments of Medicine and Physics, University of Toronto and the Toronto General Hospital.

ABSTRACT

The effects of nutritional support on body weight, nitrogen and potassium were investigated and were compared with changes in serum albumin and delayed cutaneous hypersensitivity (DCH). Fifteen malnourished patients (maintained by total parenteral nutrition (TPN) were studied for periods of 5 to 33 months. The TPN consisted of infusions of amino acids with a dual substrate of glucose and lipids. Body nitrogen was measured by prompt gamma ray analysis and potassium was determined by measurement of the naturally occurring radioactive ^{40}K in a whole body counter.

Results of sequential measurements show on average a significant increase in body nitrogen of 30% ($P < 0.001$) and a smaller but significant increase of 10% in body weight but a mean increase of 4.5% for potassium was not significant. While the nutritional support was very effective in improving body nitrogen, it was less effective in improving serum albumin or DCH.

Thus, changes in body protein are not necessarily reflected by measurements of the body parameters of nutritional status. For a reliable estimate of body protein, the direct measurement of body nitrogen is required.

INTRODUCTION

Recently, there has been a great deal of interest in evaluating malnutrition and in assessing the effect of nutrient administration on patient response. This has been done in a variety of ways. Anthropometric measurements have been advocated, to assess body fat stores and to derive the lean body mass (1). Plasma protein levels have been measured to obtain an idea of liver protein synthesis. Determinations of body potassium have been made to estimate muscle mass (2, 3). Delayed cutaneous hypersensitivity (DCH) has been proposed as a way of monitoring patients for the presence of malnutrition and for assessing recovery with nutritional support because DCH is suppressed in patients with protein-caloric malnutrition (4). More recently, procedures to measure whole body nitrogen have been developed based on prompt gamma ray analysis (5, 6) or in vivo neutron activation analysis (7). Protein mass is 6.25 times mass of nitrogen from the composition of amino acids. Our prompt gamma ray facility for body nitrogen is reproducible and reliable (8).

While these different methods are available to the clinician, they do not necessarily vary to the same degree or even in the same direction in response to malnutrition. For example, plasma protein synthesis may not correlate with total nitrogen balance. Hence it is not clear which parameters are more sensitive or suitable in the evaluation of a patient's nutritional status. The current study was undertaken to investigate the effect of nutritional support on various parameters of body composition. Malnourished patients were studied during total parenteral nutrition (TPN) consisting of the infusion of amino acids with a dual

energy substrate of glucose and lipids. Changes in body weight, serum albumin and delayed cutaneous hypersensitivity were compared with changes in total body nitrogen (TBN) and potassium (TBK) in order to assess how much these parameters are altered by such support. This has become of special importance since Yeung et al. (9), using neutron activation to measure body nitrogen, showed that weight gain during hyperalimentation with a single energy substrate, namely glucose, was due largely to increases in body water and fat.

SUBJECTS AND METHODS

Control Subjects. Healthy volunteers, 15 males and 8 females.

Patients. Fifteen malnourished patients were studied and these consisted of 8 males and 7 females with various gastrointestinal disorders necessitating home TPN. Eleven patients were studied prior to the institution of TPN and in the remaining 4 patients, the initial study was done 1 to 6 years after starting TPN (cases 2,7,10 and 15) (Table I). The study was repeated at intervals of up to 33 months after the first study.

Body Protein (Nitrogen) Technique. Body protein was measured as nitrogen by the technique of prompt gamma ray analysis. The technique has been fully described elsewhere (6). Briefly, at Toronto, the body is exposed to a flux of neutrons (produced by Pu-Be sources) which converts some of the ^{14}N in the body to ^{15}N. The ^{15}N is stable, but is created in an excited state which will immediately deexcite, emitting a 10.8 MeV gamma ray which may be detected with NaI (Tl) crystals. The number of gamma rays detected is proportional to the mass of measurements of variously sized urea-filled phantoms. The radiation to the patient is 50 mrem dose equivalent and the reproducibility of the measurements on subjects is better than 6% (8). The accuracy of the procedure was established from analysis of two pigs in which the N results by prompt gamma analysis agreed with chemical determinations to within 8%, well within the accuracy of the two methods (8).

Body Potassium Measurements. These are made by counting the naturally occurring radioisotope ^{40}K in the whole body counter as described previously (10). The ^{40}K counts have been standardized for variation in gamma efficiency with differences in body size by use of ^{42}K internal standardization. The potassium results are reproducible to $\pm$5% (1SD).

Delayed Cutaneous Hypersensitivity. This estimate was carried out by a modification of the method of McLaughlin et al. (4). Intradermal injections of 0.1 ml of each of 4 antigens were given: tuberculin purified protein derivative (1TU) (Connaught Lab. Ltd., Willowdale, Ontario), Candida (Monilia) Albicans (1/100) (Bencard Allergy Service, Weston, Ontario), mumps skin test antigen (at least 2 complement fixing units) (Eli Lily & Co., Indianopolis, Ind.) and streptokinase-streptodornase (12.5 U) (Lederle Lab., Peace River, N.Y.).

RESULTS

Initial Investigation. (See Table I). The 11 patients studied prior to commencement of TPN had a body weight $\leq$ 90% of ideal weight (using the ideal weight tables of Bistrian et al.) (11). The 4 subjects already receiving TPN were above ideal body weight. Serum albumin was low (< 3.5 g/dl) in 7 patients and normal in 8 patients. Delayed cutaneous hypersensitivity (DCH) was absent, i.e. patients were anergic, in 4 of the 11 patients in whom it was tested and serum albumin was low in 2 of these 4 patients with anergy.

The body nitrogen and potassium values of the 15 patients are shown in Table I. As with body weight, the normal TBN and TEK would be expected to vary with body size. Table II shows the mean values for the 23 volunteers and then expresses

TABLE I
Initial Clinical Data

Case No.	Sex	Age yrs	Height cm	Weight kg	%I.W.*	TBN kg	TBK g	Serum albumin g/dl	DCH**	Diagnosis
1	M	49	170.5	53.6	84	1.22	89	4.0	+	Total pancreatectomy
2#	M	43	173.7	83.5	125	1.40	118	3.6	-	Short bowel, infarct
3	M	55	171.2	56.0	86	1.27	78	3.6	-	Massive bowel resection
4	M	67	171.0	55.6	86	1.09	79	2.8	ND	Scleroderma
5	M	38	167.5	53.0	85	1.34	106	3.8	ND	Pseudo-obstruction
6	M	23	172.0	56.0	86	1.36	105	4.0	ND	Massive bowel resection
7#	M	27	181.5	81.0	112	1.76	139	4.0	+	Short bowel, infarct
8	M	37	171.2	57.0	88	1.16	98	3.9	ND	Crohn's disease and massive bowel resection
9	F	43	159.5	46.5	89	0.96	73	3.6	+	Crohn's disease with bowel obstruction
10#	F	47	165.2	62.0	111	1.26	91	2.6	-	Lipodystrophy
11	F	47	164.0	49.1	89	1.16	76	3.1	+	Short bowel, infarct
12	F	34	153.0	38.0	77	0.69	60	3.6	+	Pseudo-obstruction
13	F	65	154.0	40.0	80	1.12	59	2.6	-	Massive bowel resection
14	F	60	159.2	40.0	76	0.98	69	3.6	+	Crohn's disease and massive bowel resection
15#	F	40	171.8	67.5	112	0.94	93	3.3	+	Short bowel, infarct
M±SD	M	42.4 ±14.5	172.3 ±4.1	61.0 ±12.6	94 ±15	1.32 ±0.21	102 ±20			
M±SD	F	48.0 ±11.0	161.0 ±6.6	49.0 ±11.6	90 ±15	1.02 ±0.19	74 ±14			

*I.W. - ideal body weight
**DCH - delayed cutaneous hypersensitivity
Cases 2,7,10 and 15 received TPN treatment prior to initial study for 1-6 years

TABLE II

A. Body Composition of Volunteers (Mean ± 1SD)

Subjects - No.		Age yrs	Height cm	Weight kg	%I.W.	TBN kg	TBK g	K(g) N(kg)
Men	15	42.3 ±14.4	174.4 ±8.4	78.4 ±11.9	117.5 ±13.8	1.97 ±.53	155 ±28	80 ±10
Women	8	47.4 ±9.3	163.8 ±5.8	63.9 ±10.0	115.0 ±17.0	1.38 ±.21	96 ±14	69 ±10

B. Body Composition of Malnourished Subjects (% of Mean Volunteer Data)

			Initial Data				Final Data			
Subjects - No.		Height %	%I.W.	N	K	K/N	%I.W.	N	K	K/N
Men	8	100	99	80	67	65	96	89	85	72	83
Women	7	101	98	78	74	77	109	86	96	74	80

the mean data for the patients from Table I as a percentage of the corresponing parameters for the volunteers. On average, the heights of the patients were not significantly different (99% for males and 98% for females) from those of the volunteers, indicating similar body size; however, for patients, the body weight was only about 80% of that of the volunteers, indicating significant malnourishment. On average, the male patients had 67% TBN and 65% TBK and the female patients had 74% TBN and 77% TBK compared with mean control values. These reduced TBN and TBK values for the women are comparable to the reduction in body weight (78% of controls), but for men there was a proportionately greater loss in TBN and TBK than in body weight. All these differences from controls are highly significant ($P < 0.001$).

Effect of Total Parenteral Nutrition. The overall changes in body weight, TBN, TBK, serum albumin and DCH, after 5-33 months of TPN, are given in Table III. There were, on average, significant increases in TBN and in weight of 30% ($P < 0.001$) and 10% ($P < 0.01$) respectively. In contrast, TBK, on average, did not increase significantly ($P > 0.05$). The greater increase in TBN compared with TBK was statistically significant ($P < 0.001$). Data from sequential measurements in 3 representative cases are shown in Fig. 1 and the total change in individual subjects in Fig. 2. In individual patients, considerable variation was observed in response to TPN therapy. This may well result from the fact that the population was not homogeneous. For example, 4 patients (cases 8,3,12,4) showed significant increases in all three parameters, 5 patients (cases 9,2,15,14,1) showed significant increases in TPN with either no change or losses in TBK and 3 patients (cases 5,6,13) showed no significant increases in either TBN or TBK despite some increase in weight. For the 12 patients who showed significant (> 12%) nitrogen retention, the average protein retention over the study periods ranged from -6 to 9 g/d with a mean of 3 g/d.

Serum Albumin. The serum albumin did not rise significantly, either in the group or when patients with hypoalbuminemia (< 3.5 g/dl) were considered separately.

Delayed Cutaneous Hypersensitivity. Three of the four patients who initially had anergy developed normal reactivity during TPN, one patient remained anergic and one, who was initially reactive, became anergic. There was no clear relationship between changes in weight, TBN or TBK and the changes in serum albumin or DCH. Of the 3 anergic patients who became reactive with TPN (case 3,10,13), patient 3 showed marked increases in weight and TBN while patient 13 showed losses in TBN and TBK. Conversely, both patient 2, who remained non-reactive, and patient 9, who became non-reactive while receiving TPN, showed significant improvement in TBN and little change in TBK. All anergic patients are, at the time of writing, alive and at home.

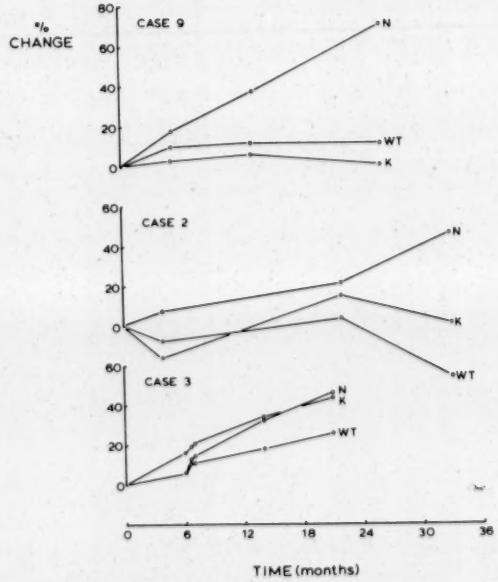

FIG. 1
Sequential measurements in 3 representative patients showing the effect of TPN therapy on body nitrogen, potassium and weight.

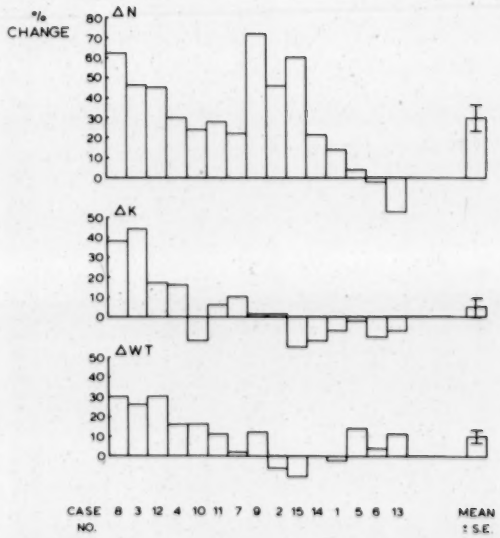

FIG. 2
Net changes in body nitrogen, potassium and weight for the 15 patients measured sequentially during TPN therapy, together with the mean changes for the total number of patients.

TABLE III
Changes (Δ) In Clinical Data During TPN Therapy

Case	Sex	Months in study	%I.W.* Initial	ΔW %	ΔN %	Mean ## protein retention g/d	ΔK %	Serum albumin Initial	Serum albumin Final	DCH** Initial	DCH** Final
1	M	5	84	-2	14	7	-7	4.0	3.6	+	ND
2#	M	33	125	-6	46	4	1	3.6	3.4	-	-
3	M	21	86	26	46	6	44	3.6	3.1	-	+
4	M	8	86	16	30	9	16	2.8	2.8	ND	ND
5	M	7	85	14	4	2	-2	3.9	4.2	ND	ND
6	M	12	86	4	-2	-5	-10	4.0	3.6	ND	+
7#	M	13	112	2	22	6	10	4.0	4.1	+	+
8	M	23	88	30	62	4	38	3.9	3.9	ND	+
9	F	26	89	12	72	6	1	3.6	3.8	+	-
10#	F	26	111	16	24	2	-12	2.6	3.0	-	+
11	F	21	89	11	28	3	6	3.1	3.1	+	+
12	F	12	77	30	45	5	17	3.8	4.2	+	+
13	F	7	80	11	-17	-6	-7	2.6	2.9	-	+
14	F	26	76	0	21	2	-12	3.6	3.5	+	+
15#	F	25	112	-10	60	5	-15	3.3	3.5	+	ND
Mean Total			92	+10	+30	3	+4.5	3.5	3.5		
±SD			±15	±12	±25	±3	± 18	±0.5	±0.5		

\# cases 2,7,10 and 15 received TPN prior to initial study for 1 to 6 years
based on average rate of nitrogen retention

DISCUSSION

The results show that malnourished women had losses of body weight, TBN and TBK of about 25%, while men showed greater loss of TBN and TBK (∿35%). After 5 to 33 months of total parenteral nutrition there were, however, significantly greater increases in total body nitrogen than in weight and total body potassium. The greater increase in TBN than in weight suggests that, in our patients, the parenteral nutrition containing lipid in addition to amino acids and glucose specifically restored lean tissue relatively more than fat tissue. In contrast, Yeung et al. (9) noted no change in TBN but a gain in water and fat when patients were given an amino acid-glucose mixture without lipid.

In the present series, there was little net increase in TBK, indicating that TBN is restored at a rate that differs from that of TBK. This finding is of importance because it shows that nitrogen retention during TPN cannot be judged by a gain in TBK, as has been done in the past. This dissociation during refeeding was noted by Collins et al (12) and Burkinshaw et al (13). The ratio of potassium (g) to nitrogen (kg) in muscle is higher than in other tissues of the body (eg. 110 g/kg in muscle compared with 50 for the skeleton, 20 for skin, 40 for fat and 52 for non-muscular soft tissue) (14). Thus, the variations in the K/N ratio seen here (Table II) could reflect differences in muscle mass. For example, one would expect K/N to decrease from 80 g/kg in a normal man to 55 in the same subject without any skeletal muscle. In support of this, we found (Table II) that normal women, who have on average relatively less muscle mass than men, have a lower K/N of 69 (K,g/N,kg). A similar sex difference in K/N was observed by Dabek et al (5). With malnutrition one would expect a loss in muscle mass and a corresponding reduction in K/N but, as seen in Table II, the malnourished patients have K/N ratios similar to those of the volunteers and, with improved nutrition as a result of TPN, the K/N ratios decreased.

These results may suggest initial restoration of protein in non-muscle lean tissue. Potassium-rich muscle may be restored more slowly or, indeed, not at all without exercise (and perhaps independently of nutrition alone). Possibly anabolizing cells take up nitrogen without influencing potassium concentration. Conversely, malnourished patients may have fluid retention with increased intracellular fluid volume and correspondingly increased total exchangeable potassium. With this situation, improved nutrition would result in fluid loss, reduction of cell volume and a corresponding reduction in potassium content. Concurrently, there would be an increase in the number of cells with the sum result of these two effects being an increase in nitrogen but little change in potassium or weight. Further investigation is required to determine the cause of these differences in potassium and nitrogen retention. The data clearly show, however, that neither body weight nor total body potassium provide a reliable estimate of the nitrogen and consequently protein, status of malnourished patients during TPN therapy.

In contrast to the profound changes in total body nitrogen (protein) the effect of nutritional support on levels of serum albumin and on DCH was less convincing. At the initial investigation, 6 of 15 patients had hypoalbuminemia and, with TPN treatment, there was no significant improvement in hypoalbuminemia. Delayed cutaneous hypersensitivity was absent in 4 of 11 patients at the time of the initial study and a fifth patient became anergic while receiving TPN. Anergy was not related to the degree of weight reduction - 2 of the anergic patients (cases 2,10) were above ideal body weight. Hypoalbuminemia was associated with anergy in 4 of 5 cases and with normal immunity in 3 of 8 cases. With TPN treatment, there was no relationship between changes in body composition (weight, TBN or TBK) and changes in DCH or concentration of serum albumin. Yeung et al (9) also reported no change in plasma proteins during TPN; they used an amino acid and hypertonic dextrose infusion and, as noted above, found no change in total body protein. In conclusion, it becomes obvious that, with nutritional support, there is very

definite improvement in total body nitrogen (protein), while the relationship of such support with levels of serum albumin or DCH is much more tentative. Furthermore, during re-alimentation, neither changes in body weight nor in body potassium are reliable indicators of changes in body protein. The most reliable method of assessing changes in body protein during re-alimentation is the direct measurement of body nitrogen. However, it remains to be shown that measurement of total body protein is the most significant test of nutritional status or only one of a number of possible tests of importance. Such assessment requires trials in different situations; the test used in these studies can be available in any hospital with a good nuclear medicine division.

Acknowledgements

The authors wish to express their appreciation to Mrs. J. Whitwell and Mrs. S Stewart for assistance in carrying out the clinical studies and in collation of the data.

This work was supported by the Bickell Foundation, The Toronto General Hospital Research Foundation and The Ontario Ministry of Health Grant No. PR.228.

REFERENCES

1. BLACKBURN, G.L., BISTRIAN, B.R., MAINI, B.S., SCHLAMM, H.T. and SMITH, M.F. Nutritional and metabolic assessment of the hospitalized patient. JPEN, 1:11-22, 1977.

2. MOORE, F.D. The Body Cell Mass and its Supporting Environment, Body Composition in Health and Disease, Philadelphia, Saunders, 1963.

3. SHIZGAL, H.M., SPANIER, A.H., HUMES, J. and WOODS, C.D. Indirect measurement of total exchangeable potassium. Am. J Physiol., 233:F253-259, 1977.

4. McLAUGHLIN, G.A., WU, A.V., SAPOROSCHETZ, I., NIMBERG, R. and MANNICK, J.A. Correlation between anergy and circulating immunosuppressive factor following major surgical trauma. Ann. Surg. 190:297-304, 1979.

5. DABEK, J.T., VARTSKY, D., DYKES, P.W., HARDWICKE, J., THOMAS, B.J., FREMLIN, J.H. and JAMES, H. Proceedings of the 5th International Conference on Modern Trends in Activation Analysis, Munich, 13-17th. September, 1976. IAEA, Vienna, p. 204.

6. MERNAGH, J.R., HARRISON, J.E. and McNEILL, K.G. In vivo determination of nitrogen using Pu-Be sources. Phys Med Biol. 22:831-835, 1977.

7. OXBY, C.B., APPLEBY, D.B., BROOKS, K., BURKINSHAW, L., KRUPOWICZ, D.W., McCARTHY, I.D., OLDRAYD, B., ELLIS, R.E., COLLINS, J.P. and HILL, G.L. A technique for measuring total-body nitrogen in clinical investigations using the ^{14}N (n,2n) ^{13}N reaction. International J of Applied Radiation and Isotopes, 29: 205-211, 1978.

8. McNEILL, K.G., MERNAGH, J.R., JEEJEEBHOY, K.N., WOLMAN, S.L. and HARRISON, J.E. In vivo measurements of body protein based on the determination of nitrogen by prompt γ analysis. Am J Clin Nutr, 32: 1955-1961, 1979.

9. YEUNG, C.K., SMITH, R.C. and HILL, G.L. Comparison of tissue composition weight changes occurring in patients fed with an elemental diet and in patients fed intravenously. Br. J. Surg, 65:816, 1978.

10. HARRISON, J.E., WILLIAMS, W.C., WATTS, J. and McNEILL, K.G. A bond calcium index based on partial-body calcium measurements by in vivo activation analysis. J. of Nuclear Medicine, 16: 116-122, 1975.

11. BISTRIAN, B.R., BLACKBURN, G.L., SHERMAN, M. and SCRIMSHAW, N.S. Therapeutic index of nutritional depletion in hospitalized patients. Surg. Gyn. Obstet, 141: 512-516, 1975.

12. COLLINS, J.P., OXBY, C.B. and HILL, G.L. Intravenous amino acids and intravenous hyperalimentation as protein-sparing therapy after major surgery. A controlled clinical trial. Lancet, 1: 788-791, 1978.

13. BURKINSHAW, L., HILL, G.L., and MORGAN, D.B. Assessment of the distribution of protein in the human body by in vivo neutron activation analysis. Nuclear Activation Techniques in the Life Sciences, IAEA, Vienna, 1979, p. 787.

14. International Committee for Radiation Protection. Report of the Task Group on Reference Man, publication 23. Pergamon Press, Oxford, 1975.

NUTRITION RESEARCH, Vol. 1, pp. 159-167, 1981
0271-5317/81/020159-09$02.00/0 Printed in the USA.
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COMPARISON BETWEEN DIETARY AND GENETIC COPPER DEFICIENCY IN MICE: COPPER-DEPENDENT ANEMIA

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ABSTRACT

Copper deficiency was produced by feeding a low copper diet to mice and rats during gestation and lactation. The copper-deficient offspring were compared to Brindled mice, which exhibit copper-deficient characteristics due to a genetic defect within the X-chromosome. Weanling mice nursed by dams fed the low copper diet exhibit classical signs of copper deficiency previously reported in other species, including: anemia, hypoceruloplasminemia, depressed brain copper levels, and cytochrome oxidase activities. However, in comparison to weanling rats derived from dams given the same treatment, the mice show only a minimal growth deficit and marginal copper depletion of liver with normal cytochrome oxidase activity. Younger 11-day-old copper-deficient male mice also develop anemia, have low tissue copper levels (liver and kidney) and exhibit decreased ceruloplasmin activity. However, unlike copper-deficient rats, these mice do not have a decrease in serum ferroxidase activity. Age-matched Brindled mice are not anemic, even though they have low serum ferroxidase activity. Similar to the dietary deficient mice, Brindled mice do have low ceruloplasmin activity and low liver copper levels. In an attempt to resolve this enigma, mouse serum was chromatographed on Sephadex G-150. Only one ferroxidase activity peak, clearly separated from ceruloplasmin, was observed. Human and rat sera display two ferroxidase activity peaks, one chromatographing with ceruloplasmin activity, which is sensitive to azide, and a second peak which, like the mouse serum peak, appears as a shoulder on the void volume. These results demonstrate the potential of this mouse model in elucidating the nature of copper-dependent anemia. Furthermore, the data from this model suggest that changes in serum ferroxidase activity cannot explain the existence of anemia in dietary copper deficiency nor absence of anemia in genetic copper deficiency (Brindled mice).

Key Words: Copper-Deficiency, Brindled Mice, Ceruloplasmin, Ferroxidase, Anemia

INTRODUCTION

The essentiality of the micronutrient, copper, was first established by demonstrating that both copper and iron were necessary to cure the anemia produced in rats fed a milk diet (1). This was the first indication of a physiological interaction between copper and iron. The precise mechanism for this interaction is unknown despite intensive studies in several species. Anemia is a constant feature of environmental copper deficiency (2).

One widely accepted theory suggests copper-dependent anemia involves reduc-

tion in ceruloplasmin (ferroxidase, EC 1.16.3.1), a cuproprotein sensitive to azide, which catalyzes the oxidation of Fe(II) to Fe(III) (3). It is suggested that lack of copper exerts its influence through ceruloplasmin by failing to mobilize stored iron from the cells to the serum where iron is normally bound to transferrin as Fe(III) (4). This theory has received experimental support from the laboratories of Frieden (5) and Cartwright (6), described elsewhere. One major criticism has been the observation that persons with Wilson's disease are not anemic, despite hypoceruloplasminemia. The enigma seemed to be resolved when Topham and Frieden (7) described the presence of a second cuproprotein (ferroxidase-II) in serum, which could oxidize iron in the presence of azide and was present in sera of patients with Wilson's disease. However, Williams et al. (8) feel that only ceruloplasmin functions as a ferroxidase in vivo and that ferroxidase II in serum can be accounted for by citrate and ferroxidase I (ceruloplasmin) which is not completely inhibited by azide. The ferroxidase activity in serum needs further characterization.

Another notable exception to the ceruloplasmin (ferroxidase I) theory of copper-dependent anemia is the absence of anemia in children with Menkes' disease (9). As in other species, human infants with environmental copper deficiency are anemic (2). Hypoceruloplasminemia is a characteristic feature of both conditions and, thus, an enigma exists.

Many of the features of Brindled mice are similar to those observed in Menkes' disease in humans (10), including X-linked inheritance and widespread symptoms characteristic of copper deficiency. Despite low levels of ceruloplasmin (diamine oxidase activity), the Brindled mice, like humans with Menkes' disease, are not anemic (11).

Brindled mice will be a useful model in resolving the enigma concerning copper-dependent anemia between environmental and genetic copper deficiency. In this paper, Brindled mice are compared with genotypically normal mice of the same inbred strain made copper-deficient by dietary treatment. Certain key aspects of copper deficiency were also studied in rats for comparison and verification of the mouse model.

METHODS

Animals and Diet. The colony of mice used to investigate environmental and genetic copper deficiency was derived from six breeding pairs purchased from Jackson Laboratories. Heterozygous females of the inbred strain C3H/HeJ carrying the Brindled allele at the Mottled locus of the X-chromosome ($Mo^{br/+}$) were mated to normal males ($Mo^{+/y}$). Brindled males ($Mo^{br/y}$) produced from these matings were compared with some of their normal brothers ($Mo^{+/y}$)--genetic copper deficiency. Female carriers ($Mo^{br/+}$) that were produced were used to expand the colony. Normal females ($Mo^{+/+}$) derived from these matings were placed with unaffected males ($Mo^{+/y}$) when they were two months old and fed a purified diet low in copper to produce dietary copper deficiency. Half of the normal females received deionized water to drink (-Cu group), the other half received water supplemented with copper sulfate, 10 µg Cu/ml (+Cu group). Male offspring (+Cu, -Cu) from this dietary study were compared to age-matched mice ($Mo^{br/y}$, $Mo^{+/y}$) from the genetic study. Sperm-positive albino rats, Sprague-Dawley (Madison, WI), weighing 200 g, were placed on the low copper diet within one day of their putative pregnancy and were given either deionized water (-Cu) or this water supplemented with copper (+Cu) to drink. Offspring were examined at various ages to compare with previous studies on dietary copper deficiency (12).

The low copper diet fed to the normal mice and rats is a purified diet, modified to omit $CuCO_3$ from the salt mix. By our analysis, the diet contains 0.5 mg Cu/kg and 45 mg Fe/kg. The composition and evaluation of the AIN-76 diet for mice has been previously described (13). With $CuCO_3$ added, the estimated copper content of the AIN-76 diet would be 6 mg Cu/kg. The deionized water given to the -Cu group contains 0.08 ng Cu/ml. Breeding units used to produce Brindled

mice were fed a non-purified diet, mouse chow (Ralston Purina), which contains 12 mg Cu/kg and 214 mg Fe/kg by analysis, and they were given tap water (50 ng Cu/ml) to drink.

Chemical Analyses. Total copper and iron were determined by flame atomic absorption spectroscopy (Perkin-Elmer model 2380) following wet-ashing of the fresh tissues and diet in HNO_3. This digestion and analytical procedure was verified by analysis of a certified standard, NBS 1577 bovine liver. Protein was measured according to a modification of the Lowry procedure (14) using bovine serum albumin as a standard. Hemoglobin was determined spectrophotometrically as cyanmethemoglobin at 540 nm using Drabkin's reagent (15).

Enzyme Assays. Following decapitation, blood was collected in acid-washed tubes and a small aliquot was removed for hemoglobin determination. The remainder was allowed to clot and the serum was removed for enzyme assays. Ceruloplasmin activity (diamine oxidase) was measured by using o-dianisidine as a substrate (16). Serum, 0.02 ml, was added to three tubes, each containing 0.18 ml of 0.1 M sodium acetate (pH 5.0). Smaller volumes of serum were used when 11-day-old mice were studied. The reaction was carried out at 30°C and initiated by adding 0.05 ml of the o-dianisidine reagent, 2.5 mg/ml of o-dianisidine dihydrochloride (Sigma Chemical Co.). The reaction was terminated by addition of 0.5 ml of 18 N H_2SO_4, and the absorbance of the oxidation product was determined at 540 nm, $\varepsilon = 9.6 \times 10^3$ ℓ $mol^{-1}cm^{-1}$. One reaction tube was stopped after 10 min and treated as a blank. The other two were stopped after 60 min. The enzymatic rate is linear between 10-60 min and the activity of ceruloplasmin is expressed as μmoles min^{-1} (liter of serum)$^{-1}$. In some studies, ceruloplasmin activity was followed by measuring the oxidation of p-phenylenediamine at 533 nm.

Serum was also used to measure total ferroxidase activity (no azide) according to the reaction conditions of Topham and Frieden (7) at 30°C and pH 6.0. The final reaction volume was reduced to 0.2 ml and initiated by adding Fe(II), which was prepared in oxygen-free water from $Fe(NH_4)_2(SO_4)_2 \cdot 6\ H_2O$ (Matheson, Coleman, and Bell). The rate of ferritransferrin formation was monitored at 460 nm, $\varepsilon = 2.5 \times 10^3$ ℓ $mol^{-1}cm^{-1}$ (Beckman Acta VI). Activity is expressed as μmol min^{-1} (liter of serum)$^{-1}$. The human apotransferrin (Sigma) was dialyzed against 0.1 mM EDTA and five changes of deionized water before use.

Brains and livers were quickly removed from the mice and rats and homogenized in 9 volumes of 0.32 M sucrose and assayed without storage for cytochrome c oxidase activity by spectrophotometrically following the loss of ferrocytochrome c at 550 nm as described previously (12).

Gel Filtration. Sephadex G-150 was equilibrated with 0.05 M sodium acetate (pH 6.8), packed and then calibrated, using blue dextran to find the void volume and with the following protein markers to estimate the molecular weights of serum ceruloplasmin and ferroxidase: glucose oxidase (A. niger); alkaline phosphatase (E. coli); serum albumin (bovine); ovalbumin (hen); and myoglobin (equine heart). These protein markers were purchased from Sigma Chemical Co.

RESULTS

It was necessary to establish that the modified AIN-76 diet could produce copper-deficient (-Cu) offspring and that copper supplementation (+Cu), 10 ppm Cu, in the drinking water was sufficient to replace the copper omitted from the salt mix. Since dietary copper deficiency had not previously been studied in mice, Sprague-Dawley rats were first used to test the dietary procedures. Pregnant rats (+Cu, -Cu) had a normal parturition and nursed their pups with no observable difficulties. When the offspring were 21 days of age, several were sampled to establish their copper and iron status (Table I). The +Cu rats appear normal in all respects; their body weight, tissue copper levels, and cytochrome oxidase activities are in good agreement with earlier studies using a similar diet based on sucrose, casein and corn oil (12). The -Cu rats are indeed copper-deficient; compared to the +Cu rats, there is a significant decrease in body weight (37%), hemoglobin (45%), ceruloplasmin activity (99%),

liver copper (96%), liver cytochrome oxidase activity (63%), brain copper (63%) and brain cytochrome oxidase activity (68%) (Table I).

TABLE I

Effects of Dietary Copper Deficiency on Rats and Mice

Parameter	Rat Offspring[1]		Mouse Offspring[1]	
	+Cu (8)	-Cu (11)	+Cu (6)	-Cu (7)
Body weight (g)	51±6.3	32±7.2*	7.48±0.73	6.61±0.36
Hemoglobin (g/dℓ)	9.00±2.49	4.91±0.39*	11.2±0.28	8.61±0.60*
Ceruloplasmin (U/ℓ)	54.1±4.32	0.27±0.51*	12.6±0.77	1.49±0.45*
Ferroxidase (U/ℓ)	110±22.2	37.2±11.5*	---	---
Liver Cu (µg/g)	20.2±6.41	0.735±0.267*	7.80±1.69	3.16±0.18*
Liver Cytochrome Oxidase (U/mg)	0.468±0.013	0.128±0.0425*	0.769±0.036	0.772±0.048
Brain Cu (µg/g)	2.06±0.11	0.557±0.117*	2.88±0.20	0.628±0.023*
Brain Cytochrome Oxidase (U/mg)	0.453±0.040	0.143±0.050*	0.557±0.049	0.208±0.015*

[1]Values are means ±S.D. for the indicated number of three-week-old animals, representing random samples taken from two litters raised at different times. Pregnant rats and mice were fed a low copper diet (0.5 mg/kg) throughout gestation and lactation. The +Cu offspring refer to animals from dams given supplemental copper (10 µg Cu/ml) in their drinking water. A * indicates that means are different ($P < 0.01$) when compared by Student's *t*-test. Analytical procedures are described elsewhere.

The dietary treatment (+Cu, -Cu) was extended to normal C3H/HeJ mice, and the offspring were also sampled at 21 days of age. Parturition and lactation appeared normal. The +Cu mice that were sampled appeared to have normal copper functions, such as ceruloplasmin activity, liver and brain copper levels, and cytochrome oxidase activities (Table I), when compared to 21-day-old mice of another strain, B6C3H, fed the non-purified diet containing 12 ppm Cu (17). When the +Cu mice are compared to mice from unsupplemented dams, -Cu, several new observations are evident (Table I). The -Cu mice have an insignificant growth depression (12%) and, in general, less severe copper deficiency than -Cu rats. However, significant decreases in hemoglobin (23%), ceruloplasmin activity (88%), liver copper (60%), brain copper (78%), and brain cytochrome oxidase activity (63%) are observed in the -Cu mice (Table I). Interestingly, the liver cytochrome oxidase activity is not lower in -Cu mice despite a significant decrease in liver copper (Table I). Thus, the brain of developing mice appears to be more susceptible to copper deficiency than the liver.

The dietary model in mice was extended to a study of younger male mice ($Mo^{+/y}$), which were derived from +Cu and -Cu dams, to compare with Brindled mice ($Mo^{br/y}$). One striking difference between genetic and dietary copper deficiency is the growth deficit (61%) observed in Brindled mice compared to controls ($Mo^{+/y}$), while the -Cu offspring, once again, showed no growth impairment (Table II). Lack of pigmentation was most noticeable in $Mo^{br/y}$ mice, although -Cu mice

did have a lighter coat color. $Mo^{br/y}$ mice die at about 2 weeks of age, whereas the -Cu mice are much more viable, and many survive when weaned and fed the low copper diet. In fact, they improve with age. Thus, there are some fundamental differences between dietary and genetic copper deficiency.

TABLE II

Selected Comparisons Between Dietary and Genetic Copper Deficiency in Mice

Parameter	11-Day-Old Male Offspring[1]			
	+Cu (11)	-Cu (11)	$Mo^{+/y}$ (15)	$Mo^{br/y}$ (11)
Dam Genotype	$Mo^{+/+}$		$Mo^{br/+}$	
Diet[2]	Modified AIN-76		Mouse Chow	
Body Weight (g)	7.12±0.40[a]	7.53±0.16[a]	7.31±0.91[a]	2.82±0.52[b]
Hemoglobin (g/dℓ)	10.5±0.72[a]	8.42±0.42[b]	10.8±0.73[a]	11.0±1.13[a]
Ceruloplasmin (U/ℓ)	13.8±1.27[a]	0.87±1.11[b]	15.4±2.24[a]	1.07±0.39[b]
Ferroxidase (U/ℓ)	2579±354[a]	2394±896[a]	1690±478[b]	242±172[c]
Liver Cu (µg/g)	37.2±10.8[a]	2.30±0.47[b]	25.4±4.04[c]	3.02±0.38[d]
Liver Fe (µg/g)	26.2±6.03[a]	35.3±12.1[a]	29.0±5.29[a]	164±56[b]
Kidney Cu (µg/g)	2.74±0.62[a]	1.62±0.39[b]	2.59±0.31[a]	8.78±1.62[c]
Kidney Fe (µg/g)	16.0±3.99[a]	14.4±3.79[a]	15.5±2.82[a]	23.0±9.35[a]

[1]Values are means ±S.D. for the indicated number of mice randomly selected from 4-7 litters. Values in the same row not sharing a common superscript are significantly different ($P < 0.01$). Analytical methods are described elsewhere.

[2]The modified AIN-76 diet contains 0.5 mg Cu/kg, and the mouse chow (Ralston) contains 12 mg Cu/kg. The +Cu offspring refer to mice nursed by dams fed the low copper diet but supplemented with $CuSO_4$ in the drinking water (10 µg Cu/ml).

As indicated previously, it is known that Brindled mice do not develop an anemia, and this is verified in our study (Table II). However, the young -Cu mice do develop an anemia, as shown by decreased hemoglobin levels (20%) (Table II), similar to the weanling -Cu mice (Table I). Therefore, mice may be added to the list of species which develop a copper-dependent anemia. Both -Cu and $Mo^{br/y}$ mice exhibit greatly depressed ceruloplasmin activities, 94% and 93%, respectively (Table II).

When serum ferroxidase activities are measured on fresh serum, a surprising result is obtained (Table II). The -Cu mice, which are anemic, have normal ferroxidase activites, whereas $Mo^{br/y}$ mice, which are not anemic, have significant decreases in serum ferroxidase activity (86%). As expected, the serum ferroxidase activity in -Cu rats, which are anemic, is depressed (66%) (Table I). In mice there does not appear to be a correlation between low ferroxidase activity and anemia. Mouse serum ferroxidase activity is twenty times higher

than rat (Table I and II) and even higher than human serum when measured under the present conditions.

Tissue metal analyses also demonstrate differences between dietary and genetic copper deficiencies. While both -Cu and $Mo^{br/y}$ mice exhibit low liver copper levels compared to their controls, 94% and 88%, respectively, kidney copper levels are markedly depressed in -Cu mice (41%) while greatly elevated in $Mo^{br/y}$ mice (339%) (Table II). Kidney iron levels are not altered by either dietary or genetic copper deficiency. Liver iron is not elevated in the -Cu mice compared to +Cu, but was quite variable in the animals sampled (Table II). Liver iron is elevated in $Mo^{br/y}$ mice (Table II) but is likely the result of the growth deficit rather than copper deficiency, since small 11-day-old $Mo^{+/y}$ mice from normal dams fed the mouse chow also have high liver iron levels, 204±38 µg/g (N = 3), but are not copper-deficient; ceruloplasmin is 12.8±6.2 U/ℓ (N = 3).

The interesting results concerning the mouse serum enzymes, ceruloplasmin and ferroxidase, prompted further experiments. Azide sensitivity was determined because azide inhibits both diamine oxidase and ferroxidase activities of human and rat ceruloplasmin (5). Normal serum was assessed for ceruloplasmin activity using o-dianisidine and ρ-phenylenediamine as substrates. The activity in both rat and human serum is eliminated by inclusion of 1 mM azide; however, only two-thirds of the mouse serum activity is eliminated, even in the presence of 10 mM NaN_3. The ferroxidase activity (A_{460}) of fresh human and rat serum was strongly inhibited by addition of azide, 1 mM. However, azide has unusual effects on mouse serum ferroxidase activity. It rarely inhibits and often stimulates an increased rate at 460 nm. The effects of azide on serum ceruloplasmin and ferroxidase indicate that major differences exist between mouse and human or rat activities. Mouse serum ferroxidase activity was rapidly lost upon storage (freezing) and, therefore, fresh serum was used for all the experiments described.

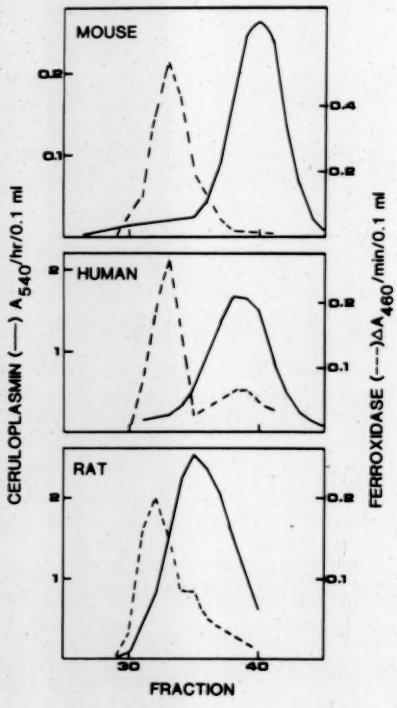

FIG. 1

Gel filtration chromatography of serum. Fresh serum (2 ml human and rat, 0.8 ml mouse) was applied to a 1.6 x 90 cm column of Sephadex G-150 equilibrated with 0.05 M sodium acetate (pH 6.8). Fractions, 2 ml each, were collected and aliquots were removed to measure enzyme activities as described elsewhere. The void volume, as determined by blue dextran elution, is fraction 31.

Serum was further investigated by gel filtration chromatography (Fig. 1). Fresh serum was chromatographed at pH 6.8 to avoid the possible generation of artifacts described by Williams et al. (8). Mouse serum has a single peak of low ceruloplasmin activity (scale expansion) with an apparent molecular weight of 115,000. The diamine oxidase activity associated with this peak is still not completely inhibited by azide. A single ferroxidase activity peak is observed eluting just after the void volume, fraction 33. It is not sensitive to azide. Human serum has one ceruloplasmin peak with an apparent molecular weight of 127,000 which is fully inhibited by azide. In contrast to mouse serum, the ferroxidase chromatographic profile from human serum shows two peaks (Fig. 1). The largest peak elutes in the same manner as mouse serum and is not azide-sensitive. The smaller peak co-chromatographs with the diamine oxidase (ceruloplasmin) peak and most likely represents ferroxidase-I, since it is sensitive to azide. When rat serum is chromatographed, one azide-sensitive ceruloplasmin peak is observed, but it has a larger apparent molecular weight, 156,000, than the peak from mouse or human. The ferroxidase profile from rat serum also exhibits two peaks of activity. Although they are not completely resolved, the smaller peak is inhibited by azide and, therefore, likely is ferroxidase-I (ceruloplasmin) as in human serum.

DISCUSSION

Environmental (dietary) copper deficiency produces anemia in mice (Table I, II) as it does in several other species (2). However, the mechanism for this copper-dependent anemia cannot be satisfied by the widely accepted ferroxidase theory (4-7), since serum ferroxidase activity is normal in -Cu mice, which have lower blood hemoglobin levels (Table II). In fact, the level of total ferroxidase activity (measured at pH 6.0) in serum of these anemic mice is higher than the activity in normal rat (18) or human (7) serum. Furthermore, total ferroxidase activity is lower in the serum of Brindled mice, and these mice are not anemic (Table II, (11)).

The role of ceruloplasmin in copper-dependent anemia is important to consider, since we have never observed an anemic -Cu mouse that did not have greatly decreased levels of ceruloplasmin (diamine oxidase) activity. However, it should be emphasized that Brindled mice, which are not anemic, exhibit low levels of serum ceruloplasmin activity (Table II, (10, 11)). We assume that mouse ceruloplasmin is a copper-dependent enzyme, since the activity in serum is lower when dietary copper is limiting, as in other species. Furthermore, its apparent molecular weight (115,000 (Fig. 1)) is similar to other purified ceruloplasmins (5), and it is partially inhibited by azide. However, since the ceruloplasmin (diamine oxidase) levels are very low in mouse serum (Tables I, II (17)) compared to human or rat, no ferroxidase-I activity was associated with it following gel filtration (Fig. 1). Mouse ceruloplasmin should be isolated to determine if it is a cuproenzyme and if it can catalyze iron oxidation.

When fresh serum is chromatographed at neutral pH, a non-ceruloplasmin ferroxidase activity peak is observed for mouse, human and rat (Fig. 1). This peak likely corresponds to ferroxidase-II described by Topham and co-workers for human (7), rat (18), and rabbit (19) serum. The high total ferroxidase activity observed in mouse serum may be due, in part, to the lack of an inhibitor naturally occurring in serum of other species, recently described (19). Mouse serum ferroxidase activity is rapidly lost upon storage, as is rabbit serum ferroxidase-II (19). If mouse serum ferroxidase activity is similar to other species, why are the -Cu mice anemic?

Topham and coworkers have presented solid evidence that ferroxidase-II from human (7), rat (18), and rabbit serum (19) is copper-dependent. Furthermore, in rats, ferroxidase-II activity falls during the course of dietary copper deficiency (18). Our own data with younger rats (Table I), however, demonstrate that anemic rats with less than 1% of the +Cu serum ceruloplasmin activity retain 34% of the +Cu serum ferroxidase activity, which is equivalent to the

normal ferroxidase-II level in rat serum (18). Since this residual ferroxidase activity is not sensitive to azide, it is, presumably, ferroxidase-II. Recently Topham has shown that purified ferroxidase-II can mobilize iron into serum in copper-deficient rabbits (20), suggesting that ferroxidase-II can act in vivo. However, in mice, serum ferroxidase activity does not correlate with the presence of anemia in -Cu mice, nor with the absence of anemia in Brindled mice.

Another theory proposed to explain copper-dependent anemia involves the role of copper in mitochondrial iron metabolism (6, 21). An intact electron transport system is required to produce Fe (II) for protoheme synthesis. Tissue cytochrome oxidase activities may indicate the functional capabilities for this process to occur. It is interesting to note that in mice cytochrome oxidase activity does not change in the same manner as in rat (Table I). Liver cytochrome oxidase activity does not fall in -Cu mice despite reduction in liver copper; however, brain cytochrome oxidase does (Table I). Brindled mice respond in exactly the same way as -Cu mice in both liver and brain (unpublished data, (22)). It will be important to measure the cytochrome oxidase activity and protoheme producing activity in other tissues from both -Cu mice and Brindled mice to determine if the enigma concerning anemia can be resolved.

This study verifies (Table II) that the Brindled mouse has low liver copper levels but elevated kidney copper levels (10, 11, 23). The -Cu mouse has low copper levels in both tissues, as expected (Table II). It appears that iron levels are not altered in liver or in kidney of either -Cu mice or Brindled mice (Table II). Brindled mice also have normal tissue levels of other trace metals (Cd, Ni, Zn) (23). In other studies (unpublished) with older -Cu mice, liver Fe levels were elevated.

Using the present model system (-Cu mice vs. Brindled mice) we were unable to define the precise etiology of copper-dependent anemia and unable to explain the absence of anemia in genetic copper deficiency (Menkes' disease and Brindled mice). However, the model system does provide us with an experimental approach to study these problems and will be useful in studying other fundamental differences between environmental and genetic copper deficiency.

ACKNOWLEDGEMENTS

The skilled technical assistance of Barbara Wilson is appreciated. This work was supported by the Minnesota Medical Foundation.

REFERENCES

1. HART, E.B., STEENBOCK, H., WADDELL, J. and ELVEHJEM, C.A. Iron in nutrition--copper as a supplement to iron for hemoglobin building in the rat. J. Biol. Chem. 77:797-812, 1928.

2. UNDERWOOD, E.J. Copper. In: Trace Elements in Human and Animal Nutrition. Academic Press, New York, 1971, pp. 57-115.

3. CURZON, G. and O'REILLY, S. A coupled iron-ceruloplasmin oxidation system. Biochem. Biophys. Res. Commun. 2:284-286, 1960.

4. OSAKI, S., JOHNSON, D.A. and FRIEDEN, E. The possible significance of the ferrous oxidase activity of ceruloplasmin in normal human serum. J. Biol. Chem. 241:2746-2751, 1966.

5. FRIEDEN, E. and HSIEH, H.S. Ceruloplasmin: the copper transport protein with essential oxidase activity. Adv. Enzymol. 44:187-235, 1976.

6. LEE, G.R., WILLIAMS, D.M. and CARTWRIGHT, G.E. Role of copper in iron metabolism and heme biosynthesis. In: Trace Elements in Human Health and Disease. A.S. Prasad (ed). Academic Press, New York, 1976, pp. 373-390.

7. TOPHAM, R.W. and FRIEDEN, E. Identification and purification of a non-ceruloplasmin ferroxidase of human serum. J. Biol. Chem. 245: 6698-6705, 1970.

8. WILLIAMS, D.M., CHRISTENSEN, D.D., LEE, G.R. and CARTWRIGHT, G.E. Serum azide-resistant ferroxidase activity. Biochim. Biophys. Acta, 350:129-134, 1974.

9. DANKS, D.M., CAMPBELL, P.E., STEVENS, B.J., MAYNE, V. and CARTWRIGHT, E. Menkes's kinky hair syndrome--an inherited defect in copper absorption with widespread effects. Pediat. 50:188-201, 1972.

10. HUNT, D.M. Primary defect in copper transport underlies mottled mutants in the mouse. Nature 249:852-854, 1974.

11. CAMAKARIS, J., MANN, J.R. and DANKS, D.M. Copper metabolism in mottled mouse mutants--copper concentrations in tissues during development. Biochem. J. 18:597-604, 1979.

12. PROHASKA, J.R. and WELLS, W.W. Copper deficiency in the developing rat brain: a possible model for Menkes' steely-hair disease. J. Neurochem. 23: 91-98, 1974.

13. Report of the American Institute of Nutrition ad hoc committee on standards for nutritional studies. J. Nutr. 107:1340-1348, 1977.

14. MARKWELL, M.A.K., HAAS, S.M., BIEBER, L.L. and TOLBERT, N.E. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Anal. Biochem. 87:206-210, 1978.

15. CARTWRIGHT, G.E. Hemoglobinometry. In: Diagnostic Laboratory Hematology. Grune & Stratton, New York, 1968, pp. 75-86.

16. LEHMAN, H.P., SCHOSINSKY, K.H. and BEELER, M.F. Standardization of serum ceruloplasmin concentrations in international units with o-dianisidine dihydrochloride as substrate. Clin. Chem. 20:1564-1567, 1974.

17. PROHASKA, J.R. Normal copper metabolism in quaking mice. Life Sci. 26: 731-735, 1980.

18. TOPHAM, R.W., SUNG, C.S.M., MORGAN, F.G., PRINCE, W.D. and JONES, S.H. Functional significance of the copper and lipid components of human ferroxidase-II. Arch. Biochem. Biophys. 167:129-137, 1975.

19. CHIN, J.L., CALISCH, M.P. and TOPHAM, R.W. Purification and characterization of the ferroxidase-II from rabbit (oryctolagus cuniculus) serum. Int. J. Biochem. 9:775-783, 1978.

20. TOPHAM, R.W., WOODRUFF, J.H., NEATROUR, G.P., CALISCH, M.P., RUSSO, R.B. and JACKSON, M.R. The role of ferroxidase-II and a ferroxidase inhibitor in iron mobilization from tissue stores. Biochem. Biophys. Res. Commun. 96: 1532-1539, 1980.

21. WILLIAMS, D.M., LOUKOPOULOS, D., LEE, G.R. and CARTWRIGHT, G.E. Role of copper in mitochondrial iron metabolism. Blood 48:77-85, 1976.

22. HUNT, D.M. Catecholamine biosynthesis and the activity of a number of copper-dependent enzymes in the copper deficient mottled mouse mutants. Comp. Biochem. Physiol. 57C:79-83, 1977.

23. HUNT, D.M. and PORT, A.E. Trace element binding in the copper deficient mottled mutants in the mouse. Life Sci. 24:1453-1466, 1979.

NUTRITION RESEARCH, Vol. 1, pp. 169-179, 1981
0271-5317/81/020169-11$02.00/0 Printed in the USA.
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NUTRIENT DEFICIENCY AND THE SENSITIVITY OF DROSOPHILA MELANOGASTER TO AN ALKYLATING AGENT

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ABSTRACT

The lowest dietary concentrations of cholesterol, choline, nicotinic acid, calcium pantothenate, pyridoxine·HCl and riboflavin that yield adult males whose longevity and fertility are sufficient for mutation studies were defined for the Canton-S strain of *Drosophila melanogaster*. Because the combined physiological indices indicated that the severities of the individual nutritional deficiencies were quantitatively similar, comparisons were made using the summed data for males fed deficient or optimal concentrations of the test nutrients. Compared to the broods of untreated control males, the incidence of unhatched eggs was higher in brood 1 of three 4-day broods and the frequencies of larval and pupal mortality were lower in all three broods derived from two day old untreated males raised on deficient diets. Dietary deficiencies apparently modify sperm function and the expression of paternal genes in the progeny. According to the frequencies of induced dominant lethal mutations, dominant lethals blocking fertilization or embryonic events were more frequent in brood 1 sperm of Trenimon-treated males raised on deficient diets than in brood 1 sperm from control-treated males; however, dominant lethals terminating larval development in brood 1, 2 and 3 progeny and pupal development in brood 1 offspring were more prevalent in the sperm of control-treated males. Lower frequencies of X-chromosomal recessive lethal mutations were found in brood 1 and 2 of deficient Trenimon-treated males than in the corresponding broods of treated-control males. Dietary deficiencies for the test nutrients are proposed to be inhibitory to the repair system that heals the broken chromosomal ends formed by Trenimon in the sperm of males of *D. melanogaster*.

Key words: mutagen, nutritional deficiency, diet, *Drosophila*.

INTRODUCTION

The nutrition of the animal is recognized as being an important variable in quantitative studies of mutagenesis, but this point is poorly documented for *Drosophila melanogaster*. Dietary differences between females have been shown to influence the gross chromosomal mutation frequency observed in X-rayed sperm (1), and a number of chemicals administered in the diet are reported to alter the sensitivity of *D. melanogaster* to different mutagenic agents. Sodium fluoride reduced the sensitivity to Trenimon and 1-phenyl-3,3-

dimethyltriazene (2,3); whereas, dietary glutathione (4) and DNA bases and base analogues (5) were protective against certain types of genetic damage due to X-irradiation. Dietary caffeine increased the incidence of detectable genetic damage when fed to females receiving irradiated sperm (6). These investigations suggest that the chromosomal repair mechanisms that operate on the gametes of Drosophila are subject to nutritional modification.

The current research was conducted to determine if deficiencies for required nutrients alter the physiological state of males of *D. melanogaster*, modifying the sensitivity of the developing gametes to mutagens.

METHODS AND MATERIALS

The Canton-S wild-type strain of *D. melanogaster* employed in this study has been examined in previous biochemical studies (7,8) and was maintained under continuous axenic culture on modified Sang's medium C for one and one-half years before the initiation of the current investigation. Test cultures were set up by placing 4 to 6 four day old female-male pairs of adults that had been raised on modified Sang's C in 6-dram shell vial cultures containing 5 ml of the test medium for 24 hours. Cultures containing 50 to 100 eggs after the removal of the adults were employed for experimental purposes. All cultures were maintained at 22.8° C with a 15 hour light - 9 hour dark lighting schedule. Cultures contaminated with microorganisms were discarded.

The composition of modified Sang's medium C is 3.5 g casein, 1.5 g agar, 1 g sucrose, 0.2 g RNA (yeast), 30 mg cholesterol, 0.03 mg biotin, 16. mg calcium pantothenate, 8 mg choline chloride, 1 mg folic acid, 1.2 mg nicotinic acid, 0.25 mg pyridoxine·HCl, 1 mg riboflavin, 0.2 mg thiamine·HCl, 25 mg $MgSO_4 \cdot 7\ H_2O$, 0.1 g $NaHCO_3$, 0.183 g KH_2PO_4, 0.189 g K_2HPO_4, 40 mg NaOH (the pH is adjusted to 6.85 with NaOH) and distilled water to a final volume of 100 ml. To identify the appropriate deficiency levels, tests were performed for at least eight concentrations of choline, cholesterol, nicotinic acid, calcium pantothenate, pyridoxine·HCl, and riboflavin ranging from an unsupplemented diet to an optimal concentration. Initially the length of the larval growth period and the percentage of larvae to survive to the pupal and adult stages were determined for each test diet. Two different concentrations of each nutrient that significantly lengthened the larval growth period and/or reduced viability were selected for further testing. Larvae were then cultured for six days on test diets containing (U-^{14}C) glucose (2×10^4 dpm/nanomole of sucrose) and the amounts of lipid and protein synthesized during the feeding periods on the deficient and control diets were determined by the methods of Geer and Downing (9). The soluble protein contents of adults raised on the deficient and control diets were also determined by the method of Lowry *et al.* (10). Ultimately, the lowest concentration of each nutrient was selected that yielded adult males with deficiency symptoms but whose longevity and fertility were sufficient for utilization in mutation studies. Because the dietary concentrations of the test nutrients required for the maximal growth and fertility of *D. melanogaster* are low compared to the required quantities of nutrients such as protein and carbohydrate, the test nutrients are collectively referred to as "micronutrients" in the text.

Two day old adult males raised on a deficient or standard synthetic diet were fed a 0.01 mM Trenimon-5% sucrose solution by the Lewis and Bacher (11) method for 17 hours, and the incidence of X-chromosomal recessive lethal mutations in the gametes in three broods determined by the *Basc* procedure by mating males to virgin 4-day old *Basc* females on the first, fourth, and eighth day after treatment (4 males to 12 females). To determine the

frequency of dominant lethal mutations two day old males raised on the test diets were fed a 0.05 mM Trenimon-5% sucrose solution for 17 hours and mated to 4-day old Canton-S females the first, fourth and eight days after treatment (one male to two females). The *Basc* and Canton-S females employed in the mutation studies were raised under uncrowded conditions on a yeast-cornmeal-sucrose-agar diet (12). Males and females were housed on the yeast-cornmeal-sucrose-agar medium during the brood mating period and the progeny were raised on this complete medium. Consequently, males were exposed to a dietary deficiency only before and during the mutagen treatment period.

Dominant lethal mutations were scored for three developmental stages. Mutations influencing fertilization or blocking embryonic events were scored as the frequency of unhatched eggs; mutations exerting a lethal influence during the larval growth period were determined as the frequency of larvae to fail to pupate (larval mortality); and pupation period lethal mutations were scored as the percentage of pupae failing to eclose (pupal mortality). For each nutrient males raised on a deficient diet and on an optimal diet were mated to *Basc* and Canton-S females to determine the control frequencies of X-chromosomal recessive lethal and dominant lethal mutations for untreated males. Comparisons between the four experimental groups of males were made to assess deficiency and deficiency-Trenimon interaction influences upon sperm function and paternal gene expression in the progeny.

Comparisons of the X-chromosomal lethal mutation data were made using the method of Kastenbaum and Bowman (13,14). The dominant lethal mutation data were subjected to Chi-square analysis (15).

Trenimon was a product of Bayer Farbenfabriken AG, Leverkusen, Deutschland. Vitamin-free casein for the test diets was obtained from the Gentosan Divison, Fisons Pharmaceutical Ltd., Leicestershire, England. Labelled compounds were acquired from Amersham/Searle Corp., Arlington Heights, Illinois. Biochemicals were purchased from the Sigma Chemical Co., St. Louis, Missouri. Other chemicals that were employed were reagent grade.

TABLE 1
Effects of Nutritional Deficiences on the Physiological State of *D. melanogaster*

Deficiency level (µg/ml)	% change from control diet[a]				
	Larval viability	Growth period	Incorporation of label into protein	Adult Protein Content	Combined Index
5.0 cholesterol	-26	-16	-6	0	-12
8.0 choline	-35	-20	-51	-18	-31
0.4 nicotinic acid	-19	-16	-58	-14	-27
1.0 calcium pantothenate	-33	-17	-68	-12	-33
0.08 pyridoxine·HCl	-18	-16	-29	-29	-23
0.4 riboflavin	-1	-38	-40	-22	-25

[a] The levels of the required nutrients in the control diet were 80 µg choline, 12 µg nicotinic acid, 10 µg riboflavin, 2.5 µg pyridoxine·HCl, 16 µg calcium pantothenate and 300 µg cholesterol per ml of medium.

RESULTS

Definition of the nutritional deficiencies. The requirements of the Canton-S strain for the nutrients examined were quantitatively very similar to those of the Oregon-S strain of *D. melanogaster* examined by Sang (16). All of the nutrients are essential for the growth and viability of the Canton-S strain but a deficiency for each nutrient exerted a slightly different influence on the growth of the animal. The characteristics that were quantified indicate different attributes of a nutritional deficiency. The length of the larval growth period indicates roughly the average larval growth rate and also the time needed to complete the events of the pupation process. Larval viability is the percentage of larvae to complete pupation and reflects the proportion of individuals irreversibly damaged by the dietary deficiency during the larval feeding period. The incorporation of label from dietary carbohydrate into protein during the first six days of the larval period is an indicator of larval growth for the initial two-thirds of the larval growth period. The soluble protein content of the adult male is a measure of the size of the adult and represents the end product of deficiency influencies. The relative changes of these characteristics for animals exposed individually to the six deficiencies compared to characteristics of animals fed an optimal diet are given in Table 1.

A choline deficiency influenced larval viability most adversely; riboflavin deficiency lengthened the larval growth period to the greatest degree; a pantothenic acid deficiency slowed the incorporation of label into protein to the greatest extent during early larval growth; and pyridoxine reduced the soluble protein content of the adult male the most. The combined indices for the four physiological characteristics suggest that except for the cholesterol deficiency, the severities of the dietary deficiencies were very similar. Cholesterol is required by *D. melanogaster* for the synthesis of ecdysone and, not surprisingly, a cholesterol deficiency exerted little influence on growth but markedly affected survival and the time required to complete the pupation process.

The frequencies of X-chromosomal recessive lethal mutations. Males of the Canton-S strain exhibited a low spontaneous rate of X-chromosomal recessive lethal mutation (Table 2). The spontaneous rates for the combined data of broods 1, 2 and 3 for untreated males raised on the control diet and untreated males raised on deficient diets were both 0.1%. Although groups of untreated males deficient for each of the test micronutrients were examined, no differences were evident between the groups.

The influences of individual dietary deficiencies on mutagen sensitivity were assessed by comparing the data for groups of deficient and control males raised in parallel and treated concurrently with Trenimon (Table 2). Although the brood 1 mutation frequencies were uniformly lower for deficient Trenimon-treated males than the brood 1 frequencies for control males, only the data for gametes from cholesterol- and riboflavin-deficient males differed significantly from the corresponding control groups. In brood 2 the mutation frequencies for pyridoxine- and riboflavin-deficient Trenimon-treated males were significantly lower than the control rates, and only the gametes derived from cholesterol- and riboflavin-deficient males exhibited significantly lower mutation frequencies than control males in brood 3. Comparisons of the combined data of broods 1, 2 and 3 for individual deficiencies with the appropriate control groups, indicated that the X-linked recessive lethal mutation frequencies in sperm from cholesterol; choline- and riboflavin-deficient Trenimon-treated males were lower than the mutation frequencies in gametes from their control-treated counterparts. The limited number of

TABLE 2
X-Chromosomal Recessive Lethal Mutation Frequencies in the Sperm of Deficient and Control Males

Diet	Brood 1			Brood 2			Brood 3		
	Chromosomes tested	lethals number	%	Chromosomes tested	lethals number	%	Chromosomes tested	lethals number	%
Trenimon-treated deficient males									
Cholesterol	1039	28	2.7[a]	1017	27	2.7	940	12	1.3[a]
Choline	1237	28	2.3	1251	19	1.5	1250	18	1.6
Nicotinic acid	1161	34	3.0	1167	20	1.7	1164	19	1.9
Pantothenic acid	1028	20	2.0	990	14	1.4	989	13	1.3
Pyridoxine	2022	49	2.5	1980	26	1.3[a]	1988	34	1.5
Riboflavin	1048	32	3.2[a]	1050	30	2.9[a]	1030	10	1.0[a]
	7535	191	2.5[b]	7455	136	1.8[b]	7361	106	1.4
Trenimon-treated control males									
Cholesterol	981	43	4.4	1022	36	3.5	1090	30	2.8
Choline	874	27	3.1	994	23	2.3	691	10	1.4
Nicotinic acid	1056	38	3.6	1053	18	1.7	1065	12	1.1
Pantothenic acid	403	11	2.7	376	6	1.6	458	6	1.3
Pyridoxine	1735	48	2.8	1515	37	2.4	2275	26	1.1
Riboflavin	462	25	5.4	465	28	6.0	439	21	4.8
	5511	192	3.5	5425	148	2.7	6018	105	1.7
Untreated deficient males	7537	5	0.1	6226	10	0.2	6218	7	0.1
Untreated control males	4813	6	0.1	4296	5	0.1	4307	6	0.1

[a]Different at the 0.05 level from the corresponding Trenimon-treated control value.

[b]Different at the 0.01 level from the corresponding Trenimon-treated control value.

significant brood differences between the sperm of Trenimon-treated males deficient for a micronutrient and control-treated males was perhaps due to the restricted sample sizes and the magnitude of the differences between the mutation frequencies of the deficient and control groups.

The possibility was entertained that different nutritional deficiencies may influence the sensitivity of *D. melanogaster* to Trenimon in the same manner. Because of the similarities of the combined physiological indices of the males raised on different deficient test diets, the deficient groups of the test males were treated as replicates for the purpose of testing the above hypothesis. Consequently, the summed data for the individual deficient-treated test groups and the control-treated test groups were compared (Table 2). The summed data for broods 1 and 2 were highly significantly different for sperm from deficient and control-treated males but the brood 3 data were similar. Apparently, late stage gametes in nutritionally deficient males were more sensitive to Trenimon-induced events leading to X-chromosomal recessive lethal mutations than were gametes of the same stages in control males.

The frequencies of dominant lethal mutations. The influence of Trenimon-treatment on egg hatchability was variable between corresponding deficiency and control groups. For brood 1 the frequencies of unhatched eggs from females mated to cholesterol-, pantothenic acid-, riboflavin- and choline-deficient males were greater than for eggs from females mated to control males; whereas, the frequencies of unhatched eggs of eggs from females mated to nicotinic acid- and pyridoxine-deficient males were less than the control values. Although the frequencies of unhatched eggs progressively declined in broods 2 and 3, the differences between deficiency and control Trenimon-treated males observed for brood 1 persisted.

The frequencies of dominant mutations lethal during the larval growth period were very consistent for control groups of males treated with Trenimon, ranging from 20.1 to 28.9 per cent. With the exception of sperm derived from pyridoxine-deficient males, the frequencies of larval period specific lethals in the sperm of Trenimon-treated nutrient-deficient males were greater than in sperm from control-treated males.

The same rationale was used for summing the dominant lethal data for the individual deficient and control groups as presented in the previous section. That is, each deficiency-control tandem is essentially a replicate for testing the hypothesis that dietary deficiencies for micronutrients effect the sensitivity of *D. melanogaster* to Trenimon in similar ways.

Micronutrient deficiencies exerted an influence on the incidence of dominant lethal mutations expressed in the progeny of test males (Table 3). The frequency of unhatched eggs was higher for the brood 1 females mated to deficient-untreated males than for females mated to control-untreated males; however, the frequencies of dominant lethals acting during the larval and pupal periods were lower in the progeny of deficient-untreated males in all three broods. The total incidence of dominant lethal mutations declined for each succeeding brood for both deficient- and control-untreated males, with deficient-untreated males exhibiting a somewhat greater frequency of lethal mutations in broods 1 and 3. Nevertheless, the decline in dominant lethal frequency in both test groups indicated that complete modified Sang's medium C may lack some nutrients that alter the paternal influence on progeny survival and that both test groups of males derived some nutritional benefit from being housed in agar-cornmeal-yeast-sucrose cultures during the brooding period.

TABLE 3
Dominant Lethal Mutation Frequencies in the Sperm of Deficient and Control Males (Summed Data)

	Deficient		Control	
	Trenimon-treated	Untreated	Trenimon-treated	Untreated
Brood 1				
No. eggs	2517	2630	2793	3081
No. larvae	919	2132	1134	2750
No. pupae	648	1835	815	2290
No. adults	595	1790	768	2192
% unhatched eggs	63.5[a]	18.9[b]	59.4	10.7
% larval mortality	29.5	13.9[b]	28.1	16.7
% pupal mortality	8.2[a]	2.5[b]	5.8	4.3
Brood 2				
No. eggs	3723	3920	3399	3510
No. larvae	1901	3483	1786	3151
No. pupae	1379	2999	1293	2656
No. adults	1329	2938	1235	2567
% unhatched eggs	48.9	11.1	47.5	10.2
% larval mortality	27.5	13.9	27.7	15.7
% pupal mortality	3.7	2.4	4.5	3.4
Brood 3				
No. eggs	3341	3549	3645	3319
No. larvae	2190	3328	2341	3049
No. pupae	1821	3032	1959	2712
No. adults	1787	2996	1894	2671
% unhatched eggs	34.5	6.2	35.8	8.1
% larval mortality	16.8	8.9[b]	16.3	11.1
% pupal mortality	1.9	1.2	3.3	1.5

[a]Different at the 0.05 level from the corresponding Trenimon-treated control group.
[b]Different at the 0.05 level from the corresponding untreated-control group.

TABLE 4
Frequencies of Induced Dominant Lethal Mutations for the Summed Data

Brood and Diet	Unhatched eggs (%)	Larval Mortality (%)	Pupal Mortality (%)
Deficient			
Brood 1	44.6[a]	15.6[a]	5.7[a]
Brood 2	37.8	13.6[a]	1.3
Brood 3	28.3	7.9[a]	0.7
Control			
Brood 1	48.7	11.4	1.5
Brood 2	37.3	12.0	1.1
Brood 3	27.7	5.2	1.8

[a]Different at the 0.05 level from the corresponding control frequency.

The data of Table 3 indicate that the brood differences in total dominant lethal frequency for deficient-untreated males were due primarily to a decline in the incidence of unhatched eggs in successive broods; whereas, the brood differences for the control-untreated males were essentially due to drops in the frequencies of larval and pupal lethals in the later broods. Thus, although the sperm of deficient-untreated males were somewhat more susceptible to dominant lethal events than sperm from control-untreated males, the most marked difference between the two groups of test males were the differing proportions of dominant lethals observed at different developmental stages.

The Trenimon-nutrient deficiency interaction was ascertainable by comparing the frequencies of induced dominant lethals (Trenimon treated frequency - spontaneous frequency x 100) for the summed data of individual deficient and control groups (Table 4). The interaction was most evident in the brood 1 data. Dominant lethal mutations that act at fertilization and embryogenesis were 4.1 per cent less frequent in deficient males because the occurrence of spontaneous events leading to unhatched eggs was 8.2 per cent more frequent in deficient-untreated males than in control-untreated males. The uncorrected incidence of dominant lethal mutations in the sperm of deficient-treated males was actually 4.1 per cent more frequent than in the sperm of control-treated males. In contrast, dominant lethals acting during the larval growth period were more frequent in all broods derived from the sperm of deficient-treated males than in the corresponding control-treated broods. Only in brood 1 was the incidence of dominant lethals expressed during the pupal period increased by the Trenimon-deficiency interaction.

DISCUSSION

In the current study the summed data for the test groups suggested that nutrient-deficient males were less susceptible to Trenimon action yielding X-chromosomal recessive lethal mutations in late and intermediate stage gametes but more prone to mutagen induced events leading to dominant lethals that block larval and pupal development. Initially these appear to be contradictory observations but the mode of action of Trenimon must be considered with the nature of X-chromosomal recessive lethal mutations and dominant lethal mutations in Drosophila. Trenimon is a trifunctional alkylating mutagen and has been characterized as a chromosomal breaking agent in *D. melanogaster* (2,3). Alkylating agents induce fewer large rearrangements than X-rays but produce an excess of small deficiences and duplications (17,18,19). An inverse relationship was observed in the changes in frequencies of small duplications and major rearrangement when the sperm of males treated with an alkylating agent were stored in females. The frequency of duplications declined while the frequency of major rearrangements increased. Both large and small rearrangements require breakage and rejoining but the opening of potential breaks that favors large rearrangements is dependent on chromosomal replication; consequently, duplications and deficiencies are formed much more rapidly than large rearrangements after chromosomes are exposed to an alkylating agent (20).

A large proportion of the X-chromosomal recessive lethals due to the action of alkylating agents are attributable to deficiencies (21,22) and dominant lethals are postulated to be largely due to chromosome breaks that fail to rejoin (23). Alkylating agents are relatively efficient producers of dominant lethals in the gametes of *D. melanogaster* compared to X-rays (24,25). Assuming that these observations are correct, X-linked recessive lethal formation by Trenimon would involve breakage and reunion; whereas, dominant lethal production would involve breakage with the retention of unhealed ends.

An exogenous factor that enhances chromosome reunion would increase the induced rate of X-chromosomal recessive lethal mutations and diminish the induced rate of dominant lethal mutations. The current hypothesis is that dietary deficiencies in the males of *D. melanogaster* inhibit the repair system that heals the broken chromosomal ends formed by Trenimon.

In the current study Trenimon induced dominant lethal mutations that were critical for larval and pupal developmental events. Whether the chromosomal events leading to "late development" dominant lethals are different than those yielding fertilization and embryonic lethals was not determined. Possibly unhealed breaks in genetically silent chromosomal regions become lethal only during the developmental period when that particular region is normally activated.

The higher incidence of unhatched eggs among the eggs oviposited by brood 1 females mated to untreated nutrient-deficient males compared to the untreated control group in the present study is of interest. Since the frequencies of larval and pupal mortality were not concordantly higher in brood 1 of the untreated-deficient males, the factor that was detrimental to egg hatchability may have been non-genetic in nature. The capacity of sperm to fertilize eggs depends upon such non-genetic factors as 1) structural features of the sperm other than DNA and 2) the quality of the seminal plasma derived from the accessory glands. A dietary deficiency might influence sperm function by modifying sperm structure or the quality of the accessory gland fluid. A requirement of accessory gland fluid for sperm maturation has yet to be shown for Drosophila but correlations between the composition of the fluid and fertility have been drawn (26,27).

ACKNOWLEDGEMENTS

We are grateful to Dr. William B. Sheridan for many helpful comments during the course of this research. The research was supported by National Institute of Environmental Health Sciences Contract No. 1-ES-5-2126.

REFERENCES

1. Herskowitz, I.H., An influence of maternal nutrition upon the gross chromosomal mutation frequency recovered from X-rayed sperm of *Drosophila melanogaster*. Genetics, 48:703-710, 1963.

2. Buchi, R., and Kurki, K., The origin of chromosome aberrations in mature sperm of *Drosophila:* Influence of sodium fluoride on treatments with Trenimon and 1-phenyl-3,3-dimethyltriazene. Arch. Genet., 48:59-67, 1975.

3. Vogel, E., Strong and antimutagenic effects of fluoride on mutation induction by Renimon and 1-phenyl-3,3-dimethyltriazene in *Drosophila melanogaster*. Mutation Res., 20:339-352, 1973.

4. Jacob, M., Radioprotective effect of six chemicals against X-ray induced genetic damage in *Drosophila melanogaster*. Mutation Res., 18:279-388, 1973.

5. Rajaramn, R., and Kamra, O.P., Modification of radiation-induced genetic damage in males germ cells of *Drosophila melanogaster* with pyrimidine nucleosides and their halogenated analogues: effects of F_1 generation. Mutation Res., 17:395-401, 1973.

6. Mendelson, D., The effect of caffeine on repair systems in oocytes of *Drosophila melanogaster*. II. The induction of chromosome aberrations in irradiated males. Mutation Res., 35:91-100, 1976.

7. Geer, B.W., Kamiak, S.N., Kidd, K.R., Nishimura, R.A., and Yemm S.J., Regulation of the oxidative NADP-enzyme tissue levels in *Drosophila melanogaster*. I. Modulation by dietary carbohydrate and lipid. J. Exp. Zool., 195:15-32, 1976.

8. Geer, B.W., and Perille, T.J., Effects of dietary sucrose and environmental temperature on fatty acid synthesis in *Drosophila melanogaster*. Insect Biochem., 7:371-379, 1977.

9. Geer, B.W., and Downing, B.C., Changes in lipid and protein syntheses during spermatozoan development and thoracic tissue maturation in *Drosophila hydei*. Wilhelm Roux Archiv. Entwicklungsmech, Org., 170:83-89, 1972.

10. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. Protein measurement with the Folin phenol reagent. J. Biol. Chem., 193: 265-275, 1951.

11. Lewis, E.B., and Bacher, E., Method of feeding ethyl methanesulfonate (EMS) to Drosophila males. Drosophila Inform. Serv., 43:193, 1968.

12. Geer, B.W., and Newburgh, R.W., Carnitine acetyltransferase and spermatozoan development in *Drosophila melanogaster*. J. Biol. Chem. 245: 71-79, 1970.

13. Kastenbaum, M.A., and Bowman, K.O., *The Minimum Number of Successes in a Binomial Sample*, ORNL-3909, Oak Ridge National Laboratory, Oak Ridge, Tenn., February, 1966.

14. Kastenbaum, M.A., and Bowman, K.O., Tables for determining the statistical significance of mutation frequencies. Mutation Res., 9:527-549, 1970.

15. Snedecor, G.W., *Statistical Methods*, 5th Edition, Iowa State University Press, Ames, Iowa. pp. 212-236, 1956.

16. Sang, J.H., The quantitative nutritional requirements of *Drosophila melanogaster*. J. Exp. Biol., 33:45-72, 1956.

17. Slizynska, H., Origin of repeats in *Drosophila* chromosomes. Genetic Res., 4:154-157, 1963.

18. Slizynska, H., The progressive approximation with storage of the spectrum of TEM-induced chromosome changes in *Drosophila* sperm to that found after irradiation. Mutation Res., 8:165-175, 1969.

19. Slizynska, H., Cytological analysis of storage effects on various types of complete and mosaic change induced in *Drosophila* chromosomes by some chemical mutagens. Mutation Res., 19:199-213, 1973.

20. Auerbach, C., *Mutation Research*, Chapman and Hall, London. pp. 256-305, 1976.

21. Bird, M.J., and Fahmy, O.G., Cytogenetic analysis of the action of carcinogens and tumor inhibitors in *Drosophila melanogaster*. I. 1:2, 3:4 - Diepoxybutane. Proc. Royal Soc. B, 140:556-578, 1953.

22. Fahmy, O.G., and Bird, M.J., Chromosome breaks among recessive lethals induced by chemical mutagens in *Drosophila melanogaster*. Heredity, 6 suppl.:149-159, 1953.

23. Loveless, A., *Genetic and Allied Effects of Alkylating Agents*, Pennsylvania State University Press, University Park, pp. 40-72, 1966.

24. Fahmy, O.G., and Fahmy, M.J., Cytogenetic analysis of the action of carcinogens and tumor inhibitors in *Drosophila melanogaster*. II. The mechanisms of induction of dominant lethals by 2:4:6 Tri(ethyleneimino)-1:3:5 Triazine. J. Genet., 52:603-619, 1954.

25. Nasrat, G.E., Kaplan, W.D., and Auerbach, C., A quantitative study of mustard gas induced chromosome breaks and rearrangements in *Drosophila melanogaster*. Z. Induktive Abstammungs - Vererbungslehre, 86:249-262, 1954.

26. Chen, P.S., and Baker, G.T., L-Alanine aminotransferase in the paragonial gland of *Drosophila*. Insect Biochem., 6:441-447, 1976.

27. Chen, P.S., and Oechslin, A., Accumulation of glutamic acid in the paragonial gland of *Drosophila nigromelanica*. J. Insect Physiol., 22:1237-1243, 1976.

NUTRITION RESEARCH, Vol. 1, pp. 181-185, 1981
0271-5317/81/020181-05$02.00/0 Printed in the USA.
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SINGLE NUTRIENT DEFICIENCY AND CELL-MEDIATED IMMUNE RESPONSES.III. VITAMIN A.

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ABSTRACT

Weanling rats were fed vitamin-A deficient diet for 3-4 weeks until the weight plateaued. The weight of the thymus and the spleen and the number of mononuclear cells contained in these lymphoid organs were similar in vitamin A-deficient animals and pair-fed controls. Lymphocyte stimulation response to phytohemagglutinin and antibody plaque forming cell number were decreased. Serum thymine hormone activity in the vitamin A-deficient group and pair-fed controls was comparable. These data confirm the important role of vitamin A in modulating cell-mediated immunity.

KEY WORDS: Vitamin A, cell-mediated immunity, thymic hormone, lymphocyte stimulation.

The relationship between nutrient intake and host resistance has been the focus of much recent investigation. Many studies have documented the occurrence of significant impairment of several aspects of immunocompetence in moderate-severe protein-energy malnutrition (1). Vitamin A deficiency, subclinical or manifest, is common in malnutrition and its presence is associated with worse prognosis (2). In laboratory animals, fed vitamin A-deficient diet, the frequency and severity of infections is increased (3). We have examined a variety of immune responses in weanling rats fed vitamin A-deficient diet for several weeks.

MATERIALS AND METHODS

Groups of weanling Sprague-Dawley rats weighing 35-40 g were fed a vitamin A-deficient diet (Tekland) for 3-4 weeks until growth plateau. Control animals were pair-fed standard laboratory diet or allowed food ad libitum supplemented with 500µg of vitamin A as all trans-retinyl acetate. The animals were housed in individual plastic cages in a room controlled for temperature (21°), humidity (40-50%) and light (alternating 12-hour periods of light and dark automatically).

Some rats were killed to determine the weight of the thymus and the spleen, the number of lymphoid cells, and to evaluate mitogen stimulation response. 1×10^6 lymphocytes separated by centrifugation on a Ficoll-Hypaque density gradient were cultured in triplicate in RPMI-1640 supplemented with 10% heat inactivated fetal calf serum and penicillin-streptomycin-fungizone. Phytohemagglutinin (PHA) was used in concentrations ranging from 0.12 to 5.0µg per well in microtiter plates. After incubation for 72 hours at 37°C in humidified air and 5% CO_2, 1 µCi of ^{3}H-thymidine was added and the plates incubated again for 12 hours. The cells were then harvested and radioactivity counted in Beckman Liquid Scintillation Counter. Results were expressed as

stimulation index defined as the ratio between counts per minute of PHA-containing cultures divided by counts per minute of cultures without PHA.

Other groups of control and vitamin A-deficient rats were immunized intraperitoneally with 2×10^8 sheep red blood cells (SRBC) and killed 5 days later to estimate the number of antibody hemolytic plaque forming cells (PFC) in the spleen employing Jerne's technique (4). 'Background' PFC were estimated in the spleens of nonimmunized animals.

Serum thymic hormone activity was quantitated by the method of Dardenne and Bach (5). The blood was allowed to clot at 4^0 temperature in plastic centrifuge tubes. The serum was separated in a refrigerated centrifuge and filtered immuniately. TF activity was assayed either the same day when the blood was collected or within a few days of storage at -20^0C. Spleen cells were obtained from 8-12-week-old male C57Bl/6J mice that had been thymectomized 10-20 days before they were killed. The spleen cells were dissociated, washed twice in Hanks solution, resuspended in a concentration of 30×10^5 cells/ml and incubated at 37^0 with serial $\log_2$ dilutions of test sera in Hanks medium and azathioprine 10 µg/ml. After 80 minutes, 1% suspension of SRBC was added. SRBC had been kept in Alsever's solution for up to 3 weeks and before use were washed two times with saline and twice with Hanks solution. The cell suspension was centrifuged at 4^0 for 5 minutes and resuspended gently. Rosettes were observed in a hemocytometer. The highest dilution of test serum sample which was able to restore azathioprine sensitivity, i.e. induce 50% inhibition of SRBC rosette-forming cells was taken as the concentration of thymic hormone activity in the serum.

Results were expressed as mean ± SD and analysed by Student's t test.

RESULTS AND DISCUSSION

Vitamin A deficiency is associated with decreased host resistance to infection. A number of factors may contribute to this susceptibility. These include epithelial metaplasia and other tissue changes. Our present study has demonstrated that vitamin A deficiency results in significant impairment of immune responses.

There was a slight decrease in the weight of the thymus and the spleen (Table 1) but this was primarily the result of associated anorexia, reduced

TABLE 1

Organ Weights

Group	Number of Animals	Thymus Weight (mg)	Spleen Weight (mg)
Vitamin A-deficient	6	181±39	317±41
Pair-fed control	6	208±28	339±47
Ad libitum control	6	281±40	387±59

nutrient intake and partial protein-calorie malnutrition. This is supported by comparable findings in pair-fed animals. We measured organ weights at an

early stage of vitamin deprivation and thus the reduction in the size of the lymphoid tissues was not as marked as that ovserved in other studies (6). The mean ±SEM lymphocyte count in the thymus and the spleen was 184±31 millions and 241±38 millions respectively in the deficient group compared with 199±29 millions and 268±43 millions respectively in the pair-fed animals. These differences are statistically not significant ($P>0.1$).

Cell number and viability were unaltered but the lymphocyte stimulation response to phytohemagglutinin was decreased (Table 2). The differences were

TABLE 2

Plaque Forming Cell Response

Group	Number of Rats	Direct PFC per spleen ($\times 10^{-3}$)	
		Background	5th day post-immunization
Vitamin A-deficient	6	0.39±0.08	17.5±4.8
Pair-fed Control	6	0.24±0.09	56.4±9.0
p		<0.05	<0.01

seen for all three concentrations of the mitogen. The impaired response may reflect a reduction in the number of responding lymphocytes or a decrease in the ability of the cell to respond. The latter may be the direct consequence of altered cell surface glycoproteins which would influence mitogen binding. Vitamin A plays a crucial role in glycoprotein synthesis. Vitamin A deficiency results in changes in a specific glycoprotein in small intestinal, corneal and tracheal epithelia (7). Our preliminary data indicate that such changes in cell surface glycoprotein also may alter the lymphocyte migration pattern in vitamin A-deficient animals.

Antibody PFC response was decreased in the animals fed vitamin A-deficient diet (Table 3). Antibody formation in response to heterologous red cells

TABLE 3

Mitogen Stimulation Response

Group	Number of Rats	PHA-Induced Stimulation Index		
		0.2 µg[a]	0.5 µg	2.0 µg
Vitamin A-deficient	6	20.7±8.8	37.5±19.0	38.7±31.9
Pair-fed Control	6	87.8±39.5	104.8±36.4	121.8±57.5
p		<0.05	<0.01	<0.01

[a]Amount of phytohemagglutinin (PHA) in each well of microtiter plate

requires an active collaboration between T and B lymphocytes and macrophages, and vitamin A deficiency may act at one or more cellular levels and their interactions.

Deficiencies of several nutrients impair the production of thymic hormones (8, 9). However, vitamin A deficiency did not alter serum thymic hormone activity other than to the extent explainable by reduction in food intake (Fig. 1).

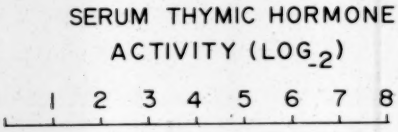

FIG. 1

Serum thymic hormone activity

This suggests that impaired cell-mediated immunity in vitamin A deficiency is not due to faulty maturation and differentiation of T lymphocytes.

Vitamin A is an essential nutrient for many metabolic processes. Its role in the visual process is widely recognized. Our studies reported here, and those of other investigators (6,10), demonstrate that dietary vitamin A is important for optimal cell-mediated immunity.

REFERENCES

1. CHANDRA, R.K. Immunology of Nutritional Disorders. Arnold, London, 1980.

2. McLAREN, D.S., SHIRAJIAN, E., TEHALIAN, M. and KHOURY, G. Xerophthalmia in Jordan. Am. J. Clin. Nutr. 17:117-130, 1965.

3. SCRIMSHAW, N.S., TAYLOR, C.E. and GORDON, J.E. Interactions of Nutrition and Infection. WHO Monograph Series 57, 1968.

4. DRESSOR, D.W. and WORTIS, H.H. Use of an antiglobulin serum for detection of plaque forming cells with low haemolytic activity. Nature, 208:859-861, 1965.

5. DARDENNE, M. and BACH, J.F. Studies on thymus products. II. Demonstration and characterization of thymic hormone. Immunology 25:353-366, 1973.

6. KRISHNAN, S., BHUYAN, U.N., TALWAR, G.P. and RAMALINGASWAMI, V. Effect of vitamin A and protein-calorie undernutrition on immune responses. Immunology 27:383-392, 1974.

7. WOLF, G. Retinol linked sugars in glycoprotein synthesis. Nutr. Rev. 35: 97-99, 1977.

8. CHANDRA, R.K., HERESI, G. and AU, B. Serum thymic factor activity in deficiencies of calories, zinc, vitamin A and pyridoxine. Clin. Exp. Immunol. 42:332-335, 1980.

9. CHANDRA, R.K., AU, B. and HERESI, G. Single nutrient deficiency and cell-mediated immune responses. II. Pyridoxine. Nutr. Res. 1:25-30, 1981.

10. NAUSS, K.M., MARK, D.A. and SUSKIND, R.M. The effect of vitamin A deficiency on the in vitro cellular immune response of rats. J. Nutr. 109:1815-1823, 1979.

NUTRITION RESEARCH, Vol. 1, pp. 187-192, 1981
0271-5317/81/020187-06$02.00/0 Printed in the USA.
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THE EFFECT OF WHEAT PROTEINS ON RAT BEHAVIOUR AND THE EFFECT OF NALOXONE HYDROCHLORIDE ON THIS RESPONSE

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ABSTRACT

When wheat proteins are fed to rats, a different form of behaviour pattern is observed by computer studies to that of unfed rats. The animals show a higher response output. After an injection of naloxone hydrochloride, this enhanced pattern is cancelled out suggesting that naloxone inhibits this behaviour pattern. It is suggested that a blocking effect takes place at either intestinal or brain receptor sites.

KEY WORDS: Wheat protein, Behaviour, Naloxone.

INTRODUCTION

It is relatively easy, in the rat,to observe abnormal patterns of behaviour after d-demphetaine administration, and also the effect of an exogenous protein on this behaviour (1,2). It is more difficult, however, to observe changes in these animals when only the exogenous protein is fed. Although these changes are slight and subtle, recording is possible by employing computer methods. It is now an accepted fact that proteins, and especially peptides,cross the intestinal barrier after oral ingestion (3,4) and it has been shown that after feeding heterologous protein to both suckling and adult rats, these proteins can be detected in brain extracts and can be precipitated by a specific immune serum(3). It has also been shown that transported proteins and fragments have an effect on behaviour patterns (1,5,6).The proposal was put forward that when intracerebral injections of gliadin polypeptides were carried out, a disruption of normal behaviour was observed with a variety of abnormal behaviour patterns being demonstrated (7,8). It was also suggested that neuroactive peptides produced during digestion of gluten affect behaviour and are environmental causes of symptoms in some schizophrenics (7).In addition to the evidence that wheat protein treatment results in changes in brain peptides, it is also possible that the effects are due to changes in other putative transmitter systems. Endorphin-like substances have been demonstrated by the action of pepsin on gliadins (9), these being naloxone reversible. These products deserve further study since the endorphins can produce marked behavioural changes. This work was carried out to see if fed wheat globulin had an effect on the normal behaviour pattern in the rat and whether this pattern was naloxone reversible.

MATERIALS AND METHODS

Animals

Male hooded rats maintained at 80% of their normal weight were used for these studies. The animals were trained for a period of eight weeks, the method used being the FI160 FT120 sequence (10). The animals used weighed 260-300 grams, were kept in home cages and given a measured amount of food which was withdrawn eight hours prior to experimentation. Water was available ad libitum. Nine animals were used for each experiment which was carried out in a Lehigh

Valley model 134-25 chamber fitted with a single lever. Control was by a solid state logic unit. Recording of data and analysis was by a Nova 1200 computer and experimental sessions were of 80 minutes duration.

Proteins

Bouquet wheat grain (Rank Hovis McDougall) was milled in a Christie hammer-mill with a 0.5mm filter attachment. The milled grain was soaked in 0.9% phosphate buffered saline (PBS) for 24 hours with constant stirring at 4°C, left to settle when the supernatant was collected and concentrated under negative pressure in a Rotavapor to give a 2% protein solution as determined by the Nessler protein estimation method. This solution containing the globulins was used for the feeding experiments. Administration of protein to rats that were lightly anaesthetised with ether was by direct cannulation into the stomach using a polyethylene cannula attached to a syringe (1). Four mls of a 2% solution was administered four hours prior to experimental sessions after which the rats were returned to their home cages. Food was withheld but water was available ad libitum. Control rats were administered four mls of a homologous rat serum at a concentration of 2%, this was used so as to give all experimental rats an equivalent amount of protein.

Drugs

Rats were injected 20 minutes prior to starting the computer sessions with either 0.2 ml water, or 0.2 ml naloxone hydrochloride (0.4 mg/ml) intraperitoneally and in muscle at the base of the neck. All animals received the same amount of naloxone/kilogram weight.

RESULTS

Figures 1-5 are the results obtained from computer studies of rats fed rat serum in PBS, wheat protein in PBS, wheat protein in PBS and an injection of

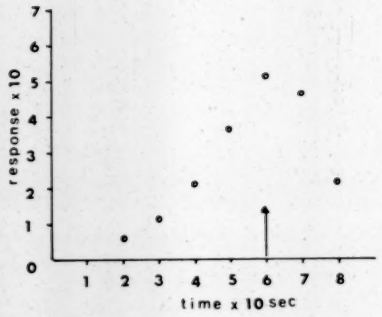


FIG. 1

Response rate output after administration of rat serum in PBS. The curve peaks at 60 seconds as expected with the schedule used.

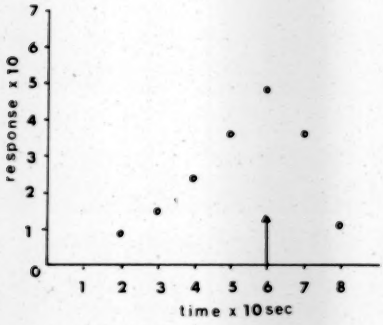


FIG. 2

Similar to Figure 1 but with a naloxone injection. The profile is very similar showing that naloxone has no effect on the control animal.

naloxone hydrochloride, and rat serum in PBS with an injection of naloxone hydrochloride. Control rats behaved as expected under experimental conditions

FIG. 3

Response rates of rats fed wheat protein. It is different from the control as it protrays a higher response rate at an earlier time, showing an enhanced pattern of behaviour.

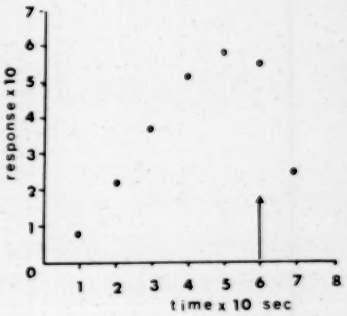
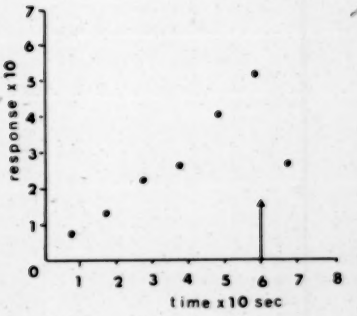


FIG. 4

Response rates after feeding wheat protein and a naloxone injection. It is almost a chiral image of Figure 2. It can be seen that naloxone has depressed the earlier pattern of behaviour.



after the administration of rat serum in PBS, response rates being similar and even in both cases. After an injection of naloxone, there was no difference in the overall response rates over an 80 minutes period. This shows that naloxone did not affect the behaviour in any way (Figures 1,2).

Rats previously fed wheat protein gave a higher activity threshold with a slight shift in the overall response pattern, this being significant in the cumulative analysis test (Figure 3). When wheat proteins were administered to rats, and the rats later injected with naloxone, an interesting observation was made in that the overall response rate was returned to control base line (Figure 4).

When these response curves were analyzed using a Hyashi Denco Auto Area meter model AAM7, control experiments gave a figure of 20, control with naloxone 21, wheat protein fed rats 39, wheat protein fed rats with a naloxone

injection 25. This bears out the analysis presented as a histogram of response output (Figure 5).

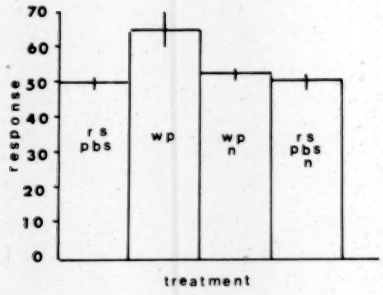


Fig. 5

The histogram represents the response rates from oral administration of rat serum in PBS, wheat protein in PBS, wheat protein in PBS + naloxone, rat serum in PBS + naloxone. Nine rats were used for each observation, statistical analysis carried out showed a significant increase with respect to rat serum and wheat protein response output activity ($p<0.05$ by the Student t test). Mean ± S.E. are given.

DISCUSSION

There is increasing evidence that the mature mammalian gut transmits macromolecules from the intestine (11), and that a large partof this transport is of large molecular weight breakdown products (4). This is of critical importance in the pathogenesis and pathophysiology of various diseases especially mental disorders. This has been well documented by Dohan (7, 8).

From this study, it can be seen that there is an enhanced behaviour pattern shown by rats after feeding wheat proteins. The only argument against these observations is that the dose administered could be considered to be of an unphysiological nature, and the fact that the rats used were underweight.

After a wheat protein challenge, the rats portray a raised response output with respect to control rats fed homologous serum protein, suggesting that the wheat proteins have an effect either at neural synaptic level, or by direct action on the brain. It has been suggested (9) that there are many exogenous peptides reaching the brain with deleterious effects. The most interesting point is the fact that after wheat protein fed rats are given an intraperitoneal injection and an injection at the base of the neck of naloxone, the raised output and temporal shift is returned to control base line,suggesting that naloxone blocks target receptor sites either at intestinal or brain level and while naloxone may be specific for opiate receptors, there is evidence suggesting that it has indirect effects on other transmitter systems, and these results do not rule out effects on other systems. It is interesting to note that these proteins have an effect on the subjects. The rats were normally fed wheat in their diet and might be expected to be equipped with intestinal IgA antibody barrier to their absorption. It may be that since they were kept underweight for quite a long period, a slight state of malnutrition arose, this being accompanied by an increase in intestinal permeability. As the schedule calls for underweight rats it was not possible to test the "normal weight" ones, as they could not have responded to the sequence. Although these response rates show up with computer

methods, they are too subtle to be recorded visually, so it could be said that the changes are not profound and it is only after the administration of amphetamine that visual behaviour changes can be monitored. The work presented is in accordance with results obtained by others (7, 10) who claim that wheat proteins do have an effect on subjects suffering from mental disorders,and that there are exogenous opioid peptides present in wheat (9). The interesting fact arising from this study is that by only feeding the wheat globulins and leaving them to normal digestion, there must be peptides formed that do have an effect on behaviour. The pattern observed was not as severe as the one observed by others (8), but a positive reaction was noted. In conclusion, it is suggested that the "toxic" or disruptive digestion products in wheat perhaps give rise to an endorphin-like substance that affects the neural pathways or the brain, and when naloxone is administered the receptor sites are blocked, thus blocking the effect of the endorphins. Whether or not the wheat protein treatment would have an effect on behaviour that has been associated with opiate effects remains to be studied.

ACKNOWLEDGMENTS

The authors are indebted to Dr. W.A. Hemmings for his continued interest in this work, and to Dr. C.F. Lowe of the Psychology Department of this University for access to the Nova computer. Thanks are also given to the Sterling Winthrop Group for gifts of Naloxone hydrochloride.

REFERENCES

1. WILLIAMS, E.W. Transport of protein by neonatal and adult rat gut. Thesis University of Wales, 1978.

2. WILLIAMS, E.W., LAIDLAW, H. and LOWE, C.F. Behaviour elicited by low doses of amphetamine after oral administration of protein to adult rats. IRCS. Med. Sci. 7:241, 1979.

3. HEMMINGS, W.A. The entry into the brain of large molecules derived from dietary protein. Proc. R. Soc. B. 200:175-192, 1978.

4. HEMMINGS, W.A. and WILLIAMS, E.W. Transport of large breakdown products of dietary protein through the gut wall. Gut 19:715-723, 1978.

5. TAYLOR, M. A preliminary investigation of dietary constituents and amphetamine induced abnormal behaviour. In:Current Research in Schizophrenia. G. Hemmings and W.A. Hemmings (eds.) MTP Press, Lancaster 1978 pp. 213-216.

6. WOODLEY, J.F, STERCHI, E.E., BRIDGES, J.F., FORSYTH, T., LUCY, J. and MAKIN, A. The digestion and absorption of dietary protein. In:The Biochemistry of Schizophrenia and Addiction. G. Hemmings (ed.) MTP Press, Lancaster, 1980, pp.277-285.

7. DOHAN, F.C. Schizophrenia, are some food derived polypeptides pathogenic? Coeliac disease as a model. In:Current Research in Schizophrenia. G. Hemmings and W.A. Hemmings (eds.) MTP Press, Lancaster, 1978, pp167-178.

8. DOHAN, F.C., LEVITT, D.R. and KUSHNIR, L.D. Abnormal behaviour after intracerebral injection of polypeptides from wheat gliadin. Pavlovian J. Biol. Sci. 12:73-82, 1978.

9. KLEE, W.A., ZIOUDROU, C. and STREATY, R.A. Exorphins. Peptides with opioid activity isolated from wheat protein and their possible role in the etiology of schizophrenia. In:Endorphins in Mental Health Research. E. Usdin, W.E. Bunney and N.S. Kline (eds.) McMillan, New York, 1979, pp.209-218.

10. HEMMINGS, W.A. Transmission of dietary protein across the gut wall and other barriers. Physiol. Rev. (in press).

NUTRITION RESEARCH, Vol. 1, pp. 193-208, 1981
0271-5317/81/020193-16$02.00/0 Printed in the USA.
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OBESITY IN INFANTS, CHILDREN, AND ADOLESCENTS IN THE UNITED STATES
II. CAUSALITY

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ABSTRACT

Investigations of the causality of obesity are broadly divided into host and environmental categories. Among host characteristics, age, sex and race appear to have no consistent effect on the prevalence of obesity among children in the United States. Reduced thermogenesis may enhance susceptibility to obesity, but the onset of the disease reflects energy intakes in excess of expenditure. Clinical conditions causing impaired regulation of energy balance are rare. The multiple environmental variables associated with obesity, primarily those within families, suggest that the factors that promote excessive energy intake for expenditure are behavioral. Although definitive proof of a causal relationship is lacking, these data suggest that the origins of obesity are environmental.

KEY WORDS: Obesity, causality, epidemiology

Obesity is rightfully viewed as an ecological phenomenon (1) caused by the interaction of a host population with its environment. Although a specific agent is the third component of the standard ecosystem of disease causality, it is invariably considered as part of the environment; agents of infectious diseases are outstanding examples. Obesity, however, has no specific agent. An energy intake in excess of expenditure is a mechanism rather than a causative agent.

The true causes of obesity are the recognizeable factors promoting excess energy intake or reduced expenditure. They include both causes of origin and causes of clinical course. Causes of origin induce disease, whereas those of course determine the nature of its progression. Both causal systems expectedly influence the natural history, aftereffects, and therapeutic response of obesity in children and adolescents.

Establishing causes of origin and clinical course of obesity is not an easy task. First, the variable considered must be demonstrably associated with disease or its progression. Second, proof of a causal relationship of the association depends on a variety of criteria (2,3): the association between the variable and obesity must be strong; a dose-effect of the variable and obesity must be shown; the association must be reproducible using a variety of approaches; exposure to the causal variable must precede the onset of disease; and, finally, the cause must explain the natural history of the disease.

We review here the host and environmental determinants of obesity, primarily those of origin, sometimes of clinical course, and occasionally those active in both directions. The first host factors considered are the innate characteristics of age, sex, and race; a second group comprises host traits

acquired either through action of other associated disease processes or by biologic or social adaptation.

HOST

Innate Characteristics

The lack of uniform criteria for the pathologic diagnosis of obesity among children and adolescents consititutes a major difficulty in assessing the effects of age, sex, or race on prevalence. To define the obese, many of the

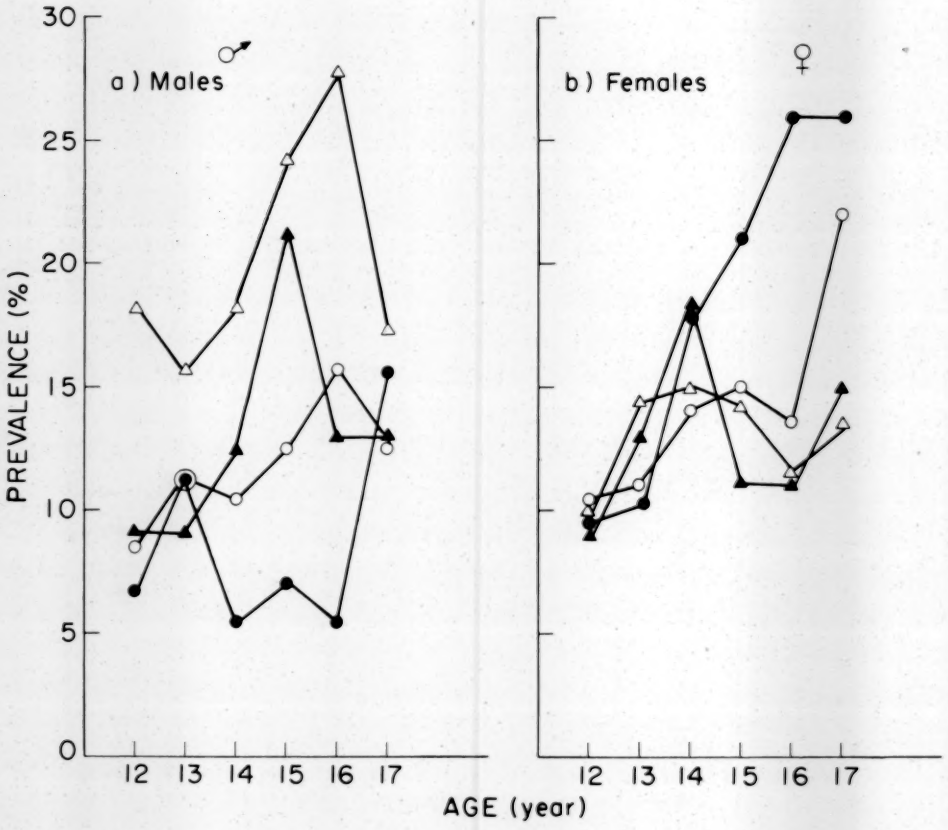

FIG. 1

Effects of age and sex on the prevalence of obesity among white (Δ——) and black (▲——) adolescent males examined in the Ten State Nutrition Survey (4) compared with white (O——) and black (●——) males examined in Cincinnati (10). Figure 1b shows similar data for white (Δ——) and black (▲——) adolescent females examined during the Ten State Nutrition Survey (4) compared with white (O——) and black (●——) adolescent females studies in Cincinnati (10). No consistent influence of age, race, or sex is apparent.

more extensive investigations (4-8) have relied on statistical criteria derived from the particular population studied, such as triceps skinfolds in excess of the 85th percentile. Such studies are useful to evaluate influence of age and sex on the natural history of fatness, yet lack of reference standards limits their utility for comparative assessment of fatness between populations. When external standards have been used (9-11), no uniform relationship of age or gender has appeared.

Figures 1a and 1b illustrate reported effects of age and sex on prevalence of obesity among white and black adolescents examined during the Ten State Nutrition Survey (TSNS) (1), and in a smaller sample restricted to residents of Cincinnati, Ohio (10). Comparison of the data from the two studies and of Figure 1a with 1b, indicates no consistent effect of age or sex. Although several investigations (6,8,10) have indicated that black children and adolescents of both sexes are leaner than their white counterparts, comparison of Figures 1a and 1b shows that racial differences are not always consistent. Socioeconomic status (4) and regional variations in fatness (8) emphasize the importance of the environment.

In addition, many comparisons assume that divisions by race assure homogeneous ethnic samples. This is obviously not true. Depending on the population sampled, racial groups conceivably include a variety of subgroups, each with differing beliefs and practices relating to energy intake and expenditure, and perhaps with a distinctive prevalence of obesity.

Data from the Midtown Manhattan Study of 1954 (12) constitute the only study in the United States of the effect of ethnicity on obesity. Females of Italian descent were more obese than those of British ancestry. Moreover, the prevalence among women was inversely related to the length of time the family had been in the United States. Both relationships were independent of socioeconomic class.

Since ethnic groups will probably be more homogenous in regard to food and exercise beliefs and practices, a comparison of existing prevalence rates among ethnic groups is an important prelude to any comparative study of causality. Furthermore, ethnicity offers substantial implications for individual therapy and community intervention. For example, among Tonganese Islanders (13) or native Hawaiians (14) where obesity is a status symbol, the obese may be particularly resistant to therapy.

Inherited Characteristics

Evidence for a genetic origin of human obesity derives from three principle sources: first, observations of twins; second, comparisons of adopted and biologic children reared in the same family; and, third, from familial patterns of fatness.

Comparisons of monozygotic and dizygotic twins show a stronger correlation of weights between monzygotic twins (15-17), particularly evident among pairs older than ten years (17). When one or both twins have subscapular skinfolds in excess of the 90th percentile, the greater average intra-pair skinfold difference occurs among dizygotic twins (16).

Correlations of triceps skinfold measurements of biologic parent-child pairs (18) are similar to those of adoptive combinations. Correlations of triceps skinfolds among biologic siblings also do not differ from those of genetically unrelated "siblings" in families with adopted children (18). Although significant correlations of mean parental weight for height in families with adopted children occurs only with nautral children (19), the correlation accounts for less than seven percent of the variance of weight for height, and may reflect no more than the heritability of height and skeletal size.

Parent-child correlations of fatness measured by triceps skinfold thickness (20) approximate $r = 0.25$ irrespective of the sex of parent or child. Also, the level of adiposity among children rises with the level of parental obesity.

Children of lean parents are generally lean, children of two obese parents tend to be obese, and children of one lean and one obese parent occupy an intermediate position, all regardless of sex (20). Sibling correlations of fatness approximate r = 0.40 and are in agreement irrespective of the particular sex (20). If one child in a family is obese, the chance of an obese sibling is 40-80% (20). Parents also resemble each other; the lowest correlation between parents' fatness is in comparison of the youngest husband-wife pairs.

Studies of twins offer convincing support for a genetic contribution to obesity. However, patterns of fatness within families, particularly the similarity of biologic and adopted children and the resemblance of genetically unrelated parents, also emphasize the importance of environment.

Congenital Disease

Several congenital syndromes in children have been associated with obesity, most commonly myelomeningocele. Clinically, close to a half of affected children are judged obese (21,22).

The Prader-Willi syndrome is characterized by obesity, hypogonadism, mild to moderate mental retardation, and small hands and feet (23-26). Early in infancy hypotonia and poor feeding are common, but are followed by a ravenous appetite in late infancy or early childhood. Mild diabetes mellitus often occurs as early as the second decade of life (27) but responds well to weight reduction (27,28). Whether diabetes under such circumstances constitutes a primary disorder of carbohydrate metabolism or an aftereffect of excessive adiposity remains uncertain. Less than one percent of patients in the Boston Children's Hospital Weight Control Program have the Prader-Willi syndrome, although this diagnosis is the most common disorder associated with obesity in that particular population.

A third syndrome associated with obesity is the Laurence-Moon-Bardet-Biedl Syndrome (29,30), characterized by obesity, mental retardation, polydactyly, hypogonadism, and retinitis pigmentosa.

As a whole, congenital syndromes associated with obesity are infrequent and readily recognized. The most frequent is probably myelodysplasia; its prevalence in Boston is between one and two per 1000 live births (31). Syndromes associated with obesity are probably offset by others leading to undernutrition such as congenital heart disease (32), and cerebral palsy (33,34). That congenital disorders contribute in any appreciable degree to the general prevalence of obesity among populations of children and adolescents appears unlikely.

Acquired Characteristics

Acquired characteristics influencing the nutritional status of the host include acute and debilitating injury, infections, and degenerative, neoplastic, or metabolic disease.

Both acute injury (35-37) and infection with fever (38) reduce energy intake and increase energy expenditure. The general result in a population is a lower prevalence of obesity.

The contribution of debilitating injury or degenerative disease to obesity warrants further investigation. In 1964, the Health Interview Survey found limitations of activity due to chronic conditions among 3.7% of individuals less than 17 years old (39). Since limited activity likely reduces energy expenditure, such disorders favor a greater prevalence of obesity in the population.

In 1901, Frohlich described a 12 year old boy with obesity, hypogonadism and a hypothalamic tumor (40). Since then, the term "Frohlich's Syndrome" has been inappropriately applied to obese male children with small genitalia, yet hypothalamic tumor as a cause of obesity is decidedly rare.

The effect of tumors, and antineoplastic therapy, is to reduce energy intake, and possibly to stimulate energy expenditure (41,42); consequently, the general effect of neoplastic disease is a lesser prevalence of obesity in

populations of children and adolescents.

Metabolic. The physiologic or metabolic causes of obesity have been intensively investigated. Nevertheless, little evidence supports these factors as responsible for any significant proportion of obesity in children and adolescents of the United States. Four general metabolic systems are considered, namely, whole body energy balance, energy source, energy regulation, and energy storage.

Energy Balance. The notion that obesity is caused by overeating is the most commonly accepted explanation of the disease. Since the mechanism for obesity is energy intake in excess of expenditure, the causes of reduced energy expenditure are equally important. Parents often reported that their obese children ate more than the non-obese (43), but the available literature lacks widespread support for this opinion. When a normal population of six-month-old North American infants was characterized by an obesity index based on weight gain, suprailiac skinfold thickness, and waist circumference, no differences in caloric intake were observed between the uppermost and lowest deciles, although children beyond the 95th percentile did consume significantly more calories than children below the 5th percentile (43). Moreover, the caloric intake of obese and overweight Swedish infants was only slightly greater than the mean intake of the study population as a whole (44). In other North American investigations, both obese male (45) and female (46,47) adolescents actually consumed fewer calories than their non-obese counterparts.

Since energy intakes among the obese and non-obese appear similar, attention has focussed on potential differences in energy expenditure. Although a complete review of that rapidly growing literature is beyond the scope of this paper, several aspects of energy expenditure pertinent to causality will be considered here.

Quantitatively, the most important component of daily energy expenditure is resting metabolic rate (RMR). Metabolic rates of obese children (48) and adults (49) are similar or may actually exceed the RMR's of the non-obese (50,51). If cellular thermogenesis accounts for up to 50% of the RMR, (52,53) then the recent report suggesting that reduced cellular thermogenesis may cause obesity appears paradoxical (54). That study reported that the activity of the red cell sodium-potassium pump was decreased in obese adults, and that the activity of the pump was inversely proportional to the degree of obesity.

However, generalizations from a study of red cells to whole body energy expenditure are unwarranted. First, other tissues were not studied. Second, activity of the pump may not account for a large proportion of the RMR (55). Third, the association of the defect with obesity could be a consequence of the disease rather than a cause. Finally, regardless of the level of energy expenditure, the onset of obesity would require an associated impairment of energy intake (56).

Other studies have considered alterations in energy expenditure in response to stimuli such as feeding, overfeeding, or physical activity. In man, consumption of food is followed by an increased metabolic rate (57). Among obese adults or children, most investigations (58-61) have shown that the thermal effects of a meal are comparable to the results observed in non-obese controls. However, at least one study found significantly greater elevations of metabolic rates among non-obese women compared to the obese (62), suggesting, perhaps, that the obese metabolize energy more efficiently.

Most experimental evidence indicates that overfeeding normal adults for more than two weeks engenders increased heat production (59). Although obese subjects have not generally shown such increases, most studies have been of short duration and used fewer excess calories for overfeeding (59).

Obese children and adolescents are measurably less active than their non-obese peers (45-47). Although activity of the obese is clearly less than that of the non-obese, the energy expenditure required for comparable activities is often greater because of the greater weight to be moved. When activity was measured directly as energy expenditure, using a 24 hour record of heart rate

correlated with oxygen consumption (63), the daily energy expenditure of obese American girls did not differ significantly from the non-obese. Likewise, although total daily energy expenditure of non-obese four and five year old children of obese parents was significantly less than that of children of non-obese parents (64), no prospective data presently link reduced energy expenditure to increased incidence of obesity.

More recently, prolonged observations of four boys with stable obesity revealed marked increases in caloric consumption and activity compared to non-obese controls (65). Since it seemed unlikely that physically inactive boys would become more active as they became obese, both the origin and persistence of their obesity was attributed to increased energy intake.

Another recent paper has suggested that obese women may have a reduced response to the thermogenic effects of norepinephrine (51). The authors of that paper noted the similarity of the response of their subjects to the reduced thermogenesis observed in genetically obese rodents, and raised the possibility of defective thermogenesis by brown fat. While their data are intriguing, several problems remain; 1) the physiologic analogue of a nonadrenaline infusion is unclear; although the authors imply that an increased metabolic efficiency occurs in obesity, this has yet to be demonstrated; 2) the contribution of brown ~~fat~~, or the potentially reduced contribution of brown fat to energy expenditure among the obese, has not yet been specified; 3) their obese subjects had increased RMR's; these would expectedly balance the reduced thermogenesis in response to norepinephrine; 4) once again, impaired regulation of food intake must be considered a necessary associated defect to account for the genesis of obesity.

Energy Source. Relatively little is known about the effect of particular sources of dietary energy on development of obesity. Proportions of dietary energy derived from fat or carbohydrate are much the same for obese male adolescents (45) or obese women (66) as for their respective controls. Furthermore, a prospective study of two groups of male infants fed isocaloric diets differing in the proportion of calories derived from fat and carbohydrate showed no differences in rates of gain in length or weight during a three month period (67).

Experimentally induced obesity (68) required fewer excess calories for weight gain, or for weight maintenance at an increased weight, when the excess calories were provided as fat rather than carbohydrate. A carbohydrate-induced increase in thermogenesis explains these observations, at least in part (69). As the cause of obesity in a general population, variations in energy source have little epidemiologic support.

Energy Regulation. Regulatory mechanisms governing food intake have been studied extensively in animals (70,71) where good evidence exists that hypothalamic lesions cause obesity. However, no evidence, clinical or otherwise, supports hypothalamic lesions as a significant cause of obesity in human populations.

Both hypothyroidism (72) and Cushing's syndrome (73) have been associated with obesity. Only one case of hypothyroidism was noted in over 1000 clinical cases of obesity at the Boston Children's Hospital Medical Center; hypothyroid children, furthermore, evidence no special tendency toward obesity (74). Since the prevalence of hypothyroidism is one per 1000 live births (75), and Cushing's syndrome is even less (76), these diseases could account for no more than a small fraction of all cases of obesity among children and adolescents.

Energy Storage. Because adults obese as children have increased numbers of adipocytes compared with persons who first became obese as adults (77,78), the public regards increased numbers of fat cells in childhood as a cause of obesity, with the added belief that periods of fat cell replication are times of increased risk.

A variety of observations suggest that this is unlikely. First, the increase of adipose cells coincides with the development of obesity; it is not the initial event (79). Replication of adipocytes occurs primarily before age

two years and during puberty (79); if periods of adipocyte replication were times of increased risk, most cases of obesity should occur prior to age two and during adolescence. In fact, epidemiologic evidence, presented in the first section of this review, suggested that development of obesity was distributed evenly throughout childhood and adolescence, with little evidence of concentration at any age.

Summary of Host Characteristics

The development of uniform standards to diagnose obesity and more refined criteria to define ethnicity will help clarify the effects of age, sex, and race on prevalence of obesity among children and adolescents in the United States.

Although a more exact contribution of acquired host disorders, such as injury, remain to be determined, it seems unlikely that observed differences will adequately account for any significant variation in prevalence. Metabolic differences, such as thermic responses to meals and to overfeeding, are more promising areas for further exploration, but currently the principle limitation is methodologic.

Caloric intakes determined either by history or observation are approximations at best. Measurements can be improved by bomb calorimetry of duplicate meals, but time and expense limit such investigations to short term studies. Activity is a poor measure of energy expenditure in the obese since it fails to reflect work required to move the added weight. Determinations of energy expenditure by cardiograms after correlating heart rate with oxygen consumption are more precise, but the coefficient of variation is still 5-10% (63,80,81). For 24-hour periods the result is a 100-200 Kcal error in an adolescent whose daily expenditure is 2000 Kcal/day. Since an intake of excess energy as low as 100 Kcal per day theoretically produces ten pounds of fat over a ten year period (49), even the best technical procedures do not clearly exclude metabolic differences related to energy balance as a cause of obesity.

Lean individuals are capable of regulating food intakes over a wide range of energy intakes. Indeed, it has been shown that the lowest energy intake of a normal 16 year old can actually be less than the maximal energy intake of a normal one year old (82). In each age group, energy intakes of normal individuals may vary by 100% (82). Therefore, even if metabolic differences are ultimately proven, one must include a second or associated defect in the regulation of energy intake as a cause of obesity. Nonetheless, differences in metabolic efficiency may account for an enhanced susceptibility to the disease.

The final problem with host causality is that most investigations published to date fail to demonstrate strong and consistent associations of any host variables with obesity. Viewed broadly, the data suggest a population specificity of prevalence that cannot be quantitated based only on frequencies of host characteristics.

ENVIRONMENT

Because energy intake and expenditure are so intimately related to the broader ecologic system affecting the host, the environment understandably plays an important role in the genesis of obesity. Clearly, prevalence is the end result of the interaction of environmental factors and those influencing host susceptibility. The particular contribution of each will vary according to existing circumstances.

Environment divides broadly into three separate classes: physical, biologic, and social. Although causal relationships of the several elements within each have not been examined in detail for obesity, the social environment, and more specifically, the family environment, stands out as the most important.

Physical Environment

Variables such as climate are easily confounded by other factors, as evidenced by the high prevalence of obesity among Alaskan Eskimo women (83) and South Pacific Tonganese Islanders (13). Nonetheless, if other variables are controlled, it is possible to examine the effects of physical factors such as altitude, or urban and rural residence on the prevalence of obesity among populations. To date, the only physical variable clearly linked to obesity is season. In suburban Boston (11) the proportion of obese school children in spring was substantially greater than in autumn.

If reduction in physical activity explains the seasonal variation in prevalence, an increase in winter activities is a reasonable target for preventive interventions. Control for season is equally important in judging the success of clinical therapy or community action programs.

Biologic Environment

The biologic environment includes all living things, plant and animal, other than man himself; particularly the prevailing food supply, infectious agents affecting man and animals, and the many features of the general environment which contribute indirectly to the welfare of both. Fluctuations in food supply, reflecting seasonal harvests, are an important determinant of rates of undernutrition in developing countries (84). In the United States, a highly developed technology for food production, processing, storage, transport, pest control, animal husbandry, and marketing has eliminated most seasonal fluctuations in the availability of main energy containing nutrients. Likewise sanitary infant foods, immunization practices, and sewage disposal have sharply reduced the incidence of many infectious diseases of man and domestic animals.

Ironically, the technologies active in control of the biologic environment may favor obesity. In the developing world, the biologic environment contributes substantially to the prevalence of undernutrition (85). However, control of the biologic environment alone does not account for the high prevalence of obesity in developed countries, since major social changes have accompanied the stabilization of the food supply and reduction of infectious disease.

Social Environment

Education. Despite the frequent use of education as a determinant of socioeconomic class, its effect on rates of obesity has rarely been examined as an independent variable. No available studies establish the relationship of parental education to prevalence of obesity among children or adolescents, although good data are at hand for adults (86). An inverse relationship of prevalence to the level of parental education carries clear implications for the design of community methods of intervention.

Economic. The effect of economic status on frequency of obesity has received much attention. Nevertheless, the two largest North American surveys that examined economic status in relation to childhood obesity presented conflicting data. The Ten State Nutrition Survey found that white children of higher socioeconomic classes were fatter than those with lower incomes, although an inverse relationship was present among girls in late adolescence (57). In contrast, a study of 3,300 white school children in three eastern cities (9) found more obesity among lower class children than at upper socioeconomic levels. Despite economic data from several other studies (7,10,43), no consistent class trends are apparent.

Existing information does little to clarify the effects of economic status on behaviors resulting in obesity. Rising prices of meat and fuel may reduce energy intake and increase physical activity, but their relationship to the prevalence of obesity has not been identified.

Family Environment

Educational, economic, and ethnic practices promote obesity among younger children through behaviors within families. In addition, family behaviors independent of these social influences will also determine energy intake and expenditure.

The effect of family environment is likely age dependent. For example, parents are primarily responsible for a four-year-old's energy intake and expenditure, but far less for that of a 15 year old. Despite the probability that familial factors have an outstanding impact on behaviors ending in obesity among preschool children, this aspect of the social environment has received limited attention. Two general categories of familial influences are considered here; namely, family dynamics, and family behaviors specifically related to energy intake and expenditure.

As emphasized earlier, the risk of childhood obesity rises with parental obesity and is independent of the sex of parent or child (20). These data alone suggest that parental influence on obesity is not simply a consequence of mothering. Both mothers and fathers (7,88,89) of obese children tend to be older than the parents of non-obese controls.

Intact parental relationships are also important. Separation from the mother has been cited as an important cause of obesity among black adolescent girls (90) and several studies have reported more frequent deaths of one parent among families of obese children (89,91-93). Since obese children can be expected to have obese parents, the latter association may reflect only an increased risk of sudden death with adult obesity rather than onset of the child's obesity in response to the parent's death (89,92). The importance of parental separation or divorce is not clear (89,92); reported differences may only reflect ethnic influences.

Both birth order and family size have been related to childhood obesity. Agreement is good that the prevalence of obesity is high among single children (88,92-95) and declines with increasing family size (92,94-96). Birth order has no consistent effect on prevalence (95) although some data suggest greater prevalence among younger children of large families (88,94).

Establishing the mechanisms that relate epidemiologic correlates of obesity to family dynamics requires a suitable methodology, not currently available, for the measurement of these interrelationships within populations. Equally important is the paucity of published studies suggesting the characteristics to be sought.

Increased parental age, single parent families, separation from the parents, single children, and younger children, all expectedly foster enhanced interdependence of parent and child. This hypothesis is strengthened by the observation of intense involvement of obese children with their families which promotes an inability of the child to develop a separate identity (97). Overprotective and possessive parental behaviors may promote overfeeding, impair the self-regulation of food intake, and cause as well as maintain obesity (97). This hypothesis has not been critically tested.

A second major category of familial behaviors includes practices relating to energy intake and expenditure. The reduced physical activity in obese adolescent girls (46,47) is characterized by a more sedentary activity at home (98) although parents of both obese and non-obese girls tend to be equally "sports-minded" (78). Aside from this report, few others relate family behavior to energy expenditure.

Decidedly more data deal with the effect of infant feeding practices on obesity among infants. Contrary to the assertion that obesity is less frequent among breast-fed than formula-fed infants (99,100), most studies have found no differences (43,101-104). Likewise, neither age at weaning (88,95), the time at which solid foods are introduced (43,102), or feeding frequency (43) have an effect. Among obese children, however, unspecified feeding difficulties were less common before four years of age (88).

Overweight children (94) and adolescents (98) eat breakfast less frequently than the non-obese, and their mothers may be less knowledgeable about nutrition and less likely to withhold desserts (43). One study indicated that mothers served their overweight boys more food than non-obese siblings with the explanation "He's a bigger boy so he needs more food (65)". However, prospective studies linking these attitudes and practices with obesity in a causal relationship have not yet been done.

The most successful current therapy for obesity is directed mainly at modifying food-related practices. Obese adults tend to choose more food and consume more food per minute than do non-obese adults (105). Obese adults also tend to be more responsive to external than to internal stimuli in the control of food intake (106,107). Although overweight children may eat more rapidly than the non-obese (65), their responsiveness to environmental cues for eating has yet to be examined.

To date no cohort studies suggest that these particular types of behavior precede the development of obesity in any age group. The influence of more readily measurable customs such as food purchasing, preparation, storage, serving and consumption also have not been examined; their effect on the prevalence or natural history of obesity remains undetermined.

Although family food-related behaviors are crucial to early nutritional status, peer group and cultural forces become more dominant with advancing age. The average North American child watches over 30 hours of television per week (108). Television watching is an obvious source of inactivity, and also influences children to consume foods of high caloric density (109-112). The association of television watching, consumption of foods advertised on television, and obesity in children has not yet been examined. Peer group practices related to food consumption or activity, or attitudes regarding body somatotype are additional variables that may affect the prevalence of obesity.

Summary of Environmental Causality

In contrast to studies of the host, several variables within the social environment have been consistently associated with obesity of children and adolescents. Of these, family relationships appear the most important. Lack of definitive proof of a causal relationship is attributable to both a limited investigation of potential behavioral mechanisms, and an inadequate methodology for measuring their importance within populations. Nonetheless it appears likely that factors within the family environment will be shown to cause obesity by promoting excessive energy intake for expenditure. However, metabolic differences may account for differences in susceptibility to obesity.

REFERENCES

1. GORDON, J.E. and LERICHE, H. The epidemiologic method applied to nutrition. Am. J. Med. Sci., 219:321-345, 1950.

2. UNITED STATES DEPARTMENT OF HEALTH, EDUCATION AND WELFARE. Smoking and Health: Report of the Advisory Committee to the Surgeon General. United States Government Printing Office. Washington, 1964.

3. SUSSER, M. Causal Thinking in the Health Sciences. Oxford University Press, New York, 1973.

4. CENTER FOR DISEASE CONTROL. Ten-State Nutrition Survey 1968-1970. Center for Disease Control (DHEW Publication No. HSM 72-8131, Atlanta, 1972.

5. HAMPTON, M.C., HUENEMAN, R.L., SHAPIRO, L.R., MITCHELL, B.W. and BEHNKE, A.R. A longitudinal study of gross body composition and body conformation and their association with food and activity in a teen-age population. Am. J. Clin. Nutr. 19: 422-435, 1966.

6. MOLINA, R.M. Patterns of development in skinfolds of Negro and White Philadelphia children. Hum. Biol., 38: 89-103, 1966.

7. FISCH, R.D., BILEK, M.K. and ULSTROM, R. Obesity and leanness at birth and their relationship to body habitus in later childhood. Pediatrics, 56: 521-528, 1975.

8. RAUH, J.L., and SCHUMSKY, D.A. An evaluation of triceps skinfold measures from urban school children. Hum. Biol., 40: 363-374, 1968.

9. STUNKARD, A., E. D'AQUILI, FOX, S. and FILLION, R.D.L. Influence of social class on obesity and thinness in children. J. Am. Med. Assoc., 221: 579-584, 1972.

10. RAUH, J.C., SCHUMSKY, D.A., and WITT, M.T. Heights, weights, and obesity in urban school children. Child. Dev., 38: 515-530, 1967.

11. JOHNSON, M.L., BURKE, B.S. and MAYER, J. The prevalence and incidence of obesity in a cross-section of elementary and secondary school children. Am. J. Clin. Nutr. 4: 231-238, 1956.

12. GOLDBLATT, P.B., MOORE, M.E. and STUNKARD, A.J. Social factors in obesity. J. Am. Med. Assoc., 192: 97-100, 1965.

13. NEWMAN, B. Are you too fat? Is dieting hopeless? Here's a spot for you. Wall Street J., 192: 1, December 5, 1978.

14. HAMMAR, S.L. Obesity and the pediatrician. Am. J. Dis. Child., 125: 787-788, 1973.

15. BAKWIN, H. Body-weight regulation in twins. Develop. Med. Child. Neurol., 15: 178-183, 1973.

16. BORJESON, M. The etiology of obesity in children. Acta. Paediatr. Scand., 65: 279-287, 1976.

17. BROOK, C.G.D. HUNTLEY, R.M.C. and SLACK, J. Influence of heredity and environment in determination of skinfold thickness in children. Brit. Med. J. 2: 719-721, 1975.

18. GARN, S.M., BAILEY, S.M. and HIGGINS, I.T.T. Fatness similarities in adopted pairs. Am. J. Clin. Nutr., 29: 1067-1068, 1976.

19. BIRON, P., MONGEAU, J.G. and BERTRAND, D. Familial resemblance of body weight and weight/height in 374 homes with adopted children. J. Pediatr., 91: 555-558, 1977.

20. GARN, S.M., and CLARK, D.C. Trends in fatness and the origins of obesity. Pediatrics, 57: 443-456, 1976.

21. RICHARD, K. BRADY, M.S. and GRESHAM, E.L. Nutritional management of the chronically ill child. Pediatr. Clin. N. Am., 24: 157-174, 1977.

22. HAYES-ALLEN, M.C. and TRING, F.C. Obesity: another hazard for spina bifida children. Brit. J. Prev. Soc. Med., 27: 192-196, 1973.

23. LAURENCE, B.M. Hypotonia, mental retardation, obesity, and cryptorchidism associated with dwarfism and diabetes in children. Arch Dis. Childh., 42: 126-139, 1967.

24. COHEN, M.M. and GORLIN, R.J. The Prader-Willi syndrome. Am. J. Dis. Child., 117: 213-218, 1969.

25. ZELLWEGER, H. and SCHNEIDER, H.J. Syndrome of hypotonia-hypomentia-hypogonadism obesity (HHHO) or Prader-Willi syndrome. Am. J. Dis. Child., 115: 588-598, 1968.

26. HALL, B.D. and SMITH, D.W. Prader-Willi syndrome. J. Pediatr., 81: 286-293, 1972.

27. EVANS, P.R. Hypogenital dystrophy with diabetic tendency. Guy's Hosp. Rep., 113: 207-22, 1964.

28. BISTRIAN, B.R., BLACKBURN, G.L. and STANBURY, J.B. Metabolic aspects of a protein-sparing modified fast in the dietary management of Prader-Willi obesity. N. Engl. J. Med., 296: 774-779, 1977.

29. BLUMEL, J., and KNIKER, W.T. Laurence-Moon-Bardet-Biedl Syndrome. Texas Reports Biol. Med., 17: 391-410, 1959.

30. SMITH, D.W. Recognizable Patterns of Human Malformation. W.B. Saunders Company, Philadelphia 1970.

31. NAGGAN, L. The recent decline in prevalence of anencephaly and spina bifida. Am. J. Epidemiol., 89: 154-160, 1969.

32. STRANGWAY, A., FOWLER, R., CUNNINGHAM, K. and HAMILTON, J.R. Diet and growth in congenital heart disease. Pediatrics, 57: 75-86, 1976.

33. BERG, K. and ISAKSSON, B. Body composition and nutrition of school children with cerebral palsy. Acta. Paediatr. Scand., Suppl. 204: 41-51, 1970.

34. CULLEY, W.J., and MIDDLETON, T.O. Caloric requirements of mentally retarded children with and without motor dysfunction. J. Pediatr., 75: 380-384, 1969.

35. CUTHBERTSON, D.P. Observations on the disturbance of metabolism produced by injury to the limbs. Quart. J. Med., 25: 233-246, 1932.

36. WILMORE, D.W., LONG, J.M., MASON, A.D., SKREEN, R.W. and PRUITT, JR., B.A. Catecholamines: mediator of the hypermetabolic response to thermal injury. Ann. Surg., 180: 653-668, 1974.

37. WILMORE, D.W. Nutrition and metabolism following thermal injury. Clin. Plastic Surg., 1: 603-619, 1974.

38. GARROW, J.S. Energy Balance and Obesity in Man. (1st Ed.) American Elsevier Pub. Co., Inc., New York, 1974.

39. NATIONAL CENTER FOR HEALTH STATISTICS. Current Estimates from the Health Interview Survey, United States, 1974. Washington: National Center for Health Statistics, DHEW # (HRA) 76-1527, 1975.

40. BRUCH, H. The Fröhlich Syndrome - report of the original case. Am. J. Dis. Child., 58: 1282-1289, 1939.

41. WATERHOUSE, C. How tumors affect host metabolism. Ann. N.Y. Acad. Sci., 230: 86-93, 1974.

42. YOUNG, V.R. Energy metabolism and requirements in the cancer patient. Cancer Res., 37: 2336-2347, 1977.

43. HUENEMANN, R.L. Environmental factors associated with preschool obesity. J. Am. Dietet. Assoc., 64: 480-487, 1974.

44. SVEGER, T., LINDBERG, T., WEIBULL, B. and OLSSON. U.L. Nutrition, overnutrition, and obesity in the first year of life in Malmö, Sweden. Acta. Paediatr. Scand., 64: 634-640, 1975.

45. STEFANIK, P.A., HEALD, F.P. JR., and MAYER, J. Caloric intake in relation to energy output of obese and non-obese adolescent boys. Am. J. Clin. Nutr., 7: 55-62, 1959.

46. JOHNSON, M.L., BURKE, B.S. and MAYER, J. Relative importance of inactivity and overeating in the energy balance of obese high school girls. Am. J. Clin. Nutr., 4: 37-44, 1956.

47. BULLEN, B.A., REED, R.B. and MAYER, J. Physical activity of obese and non-obese adolescent girls appraised by motion picture sampling. Am. J. Clin. Nutr., 14: 211-222, 1964.

48. BRUCH, H. Obesity in childhood. II. Basal Metabolism and serum cholesterol of obese children. Am. J. Dis. Child., 58: 1001-1022, 1939.

49. BRAY, G.A. The Obese Patient. W.B. Saunders Co., Philadelphia, 1976.

50. JAMES, W.P.T., BOILES, F., DAVIES, H.L., DAUNCEY, M.J. Elevated metabolic rates in obesity. Lancet, I: 1122-1125, 1978.

51. JUNG, R.T., SHETTY, P.S., JAMES, W.P.T., BARRAND, M.A. and CALLINGHAM, B.A. Thermogenesis in obesity. Nature, 279: 322-323, 1979.

52. WHITTAM, R. Activecation transport as a pace-maker of respiration. Nature, 191: 603-604, 1961.

53. WHITTAM, R. and WILLIS, J.S. Ion movements and oxygen consumption in kidney cortex slices. J. Physiol., 168: 158-177, 1963.

54. DE LUISE, M., BLACKBURN, G.L. and FLIER, J.S. Reduced activity of the red-cell sodium-potassium pump in human obesity. N. Engl. J. Med., 303: 1017-1022, 1980.

55. HIMMS-HAGEN, J. Cellular thermogenesis. Ann. Rev. Physiol., 39: 315-351.

56. BONDY, P.K. Metabolic obesity. N. Engl. J. Med., 303: 1057-1058, 1980.

57. GARROW, J.S. and HAWES, S.F. The role of amino acid oxidation in causing 'specific dynamic action' in man. Brit. J. Nutr., 27: 211-219, 1972.

58. GARROW, J.S. The regulation of energy expenditure in man. Recent Advances in Obesity Research: II. Newman Publishing Ltd. London 1978, pp. 200-210.

59. GARROW, J.S. Energy Balance and Obesity in Man. (2nd Ed.) Elsevier/ North Holland Biomedical Press, New York, 1978.

60. BRUCH, H. Obesity in childhood. IV. Energy expenditure of obese children. Am. J. Dis. Child., 60: 1082-1109, 1940.

61. STORDY, B.J., MARKS, V., KALUCY, R.S. and CRISP, A.H. Weight gain, thermic effect of glucose, and resting metabolic rate during recovery from anorexia nervosa. Am. J. Clin. Nutr., 30: 138-146, 1977.

62. PITTET, P., CHAPPIUS, P., ACHESON, K., DE TECHTERMANN, F. and JEQUIER, E. Thermic effect of glucose in obese subjects studied by direct and indirect calorimetry. Brit. J. Nutr., 35: 281-292, 1976.

63. BRADFIELD, R.B., PAULOS, J. and GROSSMAN, L. Energy expenditure and heart rate of obese high school girls. Am. J. Clin. Nutr., 24: 1482-1488, 1971.

64. GRIFFITHS, M. and PAYNE, P.R. Energy expenditure in small children of obese and non-obese parents. Nature, 260: 698-700, 1976.

65. WAXMAN, M., and STUNKARD, A.J. Caloric intake and expenditure of obese boys. J. Pediatr., 96: 1870193, 1980.

66. BEAUDOIN, R., and MAYER, J. Food intakes of obese and non-obese women. J. Am. Dietet. Assoc., 29: 29-33, 1953.

67. FOMON, S.J., THOMAS. L.N. FILER, L.J. JR., ANDERSON, T.A. and NELSON, S.E. Influence of fat and carbohydrate content of diet on food intake and growth of male infants. Acta. Paediatr. Scand., 65: 136-144, 1976.

68. SIMS, E.A.H., DANFORTH, E. JR., HORTON, E.S., BRAY, S.A., GLENNON, J.A. and SALANS, L.B. Endocrine and metabolic effects of experimental obesity in man. Rec. Prog. Hormone Res., 29: 457-496.

69. FLATT, J.P. The biochemistry of energy expenditure. In: Recent advances in Obesity Research: II. G.A. Bray (Ed.) Newman Publishing Co., London, 1978.

70. MAYER, J. Some aspects of the problem of regulation of food intake and obesity. N. Engl. J. Med., 274: 662-672, 1966.

71. MAYER, J. Genetic, traumatic, and environmental factors in the etiology of obesity. Physiol. Rev., 33: 472-508, 1953.

72. GREENE, J.A. Clinical study of the etiology obesity. Ann. Int. Med., 12: 1797-1803, 1937.

73. McARTHUR, R.G., CLOUTIER, M.D., HAYLES, A.B. and SPRAGUE, R.G. Cushing's disease in children. Mayo Clin. Proc., 47: 318-326.

74. HEALD, F.P. and KHAN, M.A. Obesity in children and adolescents. In: Endocrine and Genetic Disease of Childhood and Adolescence. (2nd Ed.) L.A. Gardner (ed.) W.B. Saunders and Co., Philadelphia 1975 pp. 1146-1173.

75. LAFRANCHI, S.H., MURPHEY, W.H., FOLEY, T.P., JR., LARSEN, P.R. and BUIST, N.R.M. Neonatal hypothyroidism detected by the Northwest regional screening program. Pediatrics, 63: 180-191, 1979.

76. KAPLAN, S.A. Disorders of the adrenal cortex I. Pediatr. Clin. N. Am. 26: 65-76, 1979.

77. HIRSCH, J. and KNITTLE, J.L. Cellularity of obese and non-obese human adipose tissue. Fed. Proc., 29: 1516-1521, 1970.

78. SALANS, L.B., KNITTLE, J.L. and HIRSCH, J. The role of cell size and adipose tissue insulin sensitivity in the carbohydrate intolerance of human obesity. J. Clin. Invest., 47: 153-165, 1968.

79. KNITTLE, J.L., TIMMERS, K. and GINSBERG-FELLNER, F. The growth of adipose tissue in children and adolescents. J. Clin. Invest., 63: 239-246, 1979.

80. BRADFIELD, R.B., HUNTZICKER, P.B. and FRUEHAN. Simultaneous comparison of respirometer and heart-rate telemetry techniques as measures of human energy expenditure. Am. J. Clin. Nutr., 22: 696-700, 1969.

81. BRADFIELD, R.B. A technique for determination of usual daily energy expenditure in the field. Am. J. Clin. Nutr., 24: 1148-1154, 1971.

82. WIDDOWSON, E. Nutritional individuality. Proc. Nutr. Soc., 21: 121-128, 1962.

83. SCHAEFER, O. Are Eskimos more or less obese than other Canadians? A comparison of skinfold thickness and ponderal index in Canadian Eskimos. Am. J. Clin. Nutr., 30: 1623-1628, 1977.

84. TROWBRIDGE, F.L. and NEWTON, L.H. Seasonal changes in nutrition and diarrheal disease among pre-school children in El Salvador. Am. J. Trop. Med. Hyg., 28: 135-141, 1979.

85. MATA, L.J. The Children of Santa Maria Cauque. M.I.T. Press, Cambridge, 1978.

86. GARN, S.M., BAILEY, S.M., COLE, P.E. and HIGGINS, I.T.T. Level of education, level of income, and level of fatness in adults. Am. J. Clin. Nutr. 30: 721-725, 1977.

87. GARN, S.M. and CLARK, D.C. Nutrition, growth, development, and maturation: findings from the Ten-State Nutrition Survey of 1968-1970. Pediatrics, 56: 306-319, 1975.

88. CRISP, A.H., DOUGLAS, J.W.B., ROSS, J.M. and STONEHILL, E. Some developmental aspects of disorders of weight. J. Psychosom. Res., 14: 313-320, 1970.

89. ASHER, P. Fat babies and fat children. Arch. Dis. Childh., 41: 672-673, 1966.

90. KAHN, E.J. Obesity in children: identification of a group at risk in a New York ghetto. J. Pediatr., 77: 771-774, 1970.

91. DINATALE, B.P., BREGAIN, A., CARBONE, B., COLACICCO, V. DELLA PORTA, and DELLAVEDOVA, B. Investigations preliminary to devising a program for prevention of childhood obesity. In: Obesity in Childhood, E. Caccion, Z. Laron, and S. Raiti (Eds.) Academic Press, New York pp. 157-163-

92. BORJESON, M. Overweight children. Acta. Paediatr. Scand., 51: Suppl. 132, 1962.

93. WILKINSON, P.W., PARKIN, J.M., PEARLSON, J., PHILIPS, P.R. and SYKES, P. Obesity in childhhood; a community study in Newcastle upon Tyne. Lancet, 1: 350-352, 1977.

94. HILLMAN, R.W. Infant feeding problems and oral habits of overweight and underweight children. Am. J. Clin. Nutr., 13: 326-330, 1963.

95. RAVELLI, G.P. and BELMONT, L. Obesity in nineteen-year-old men: family size and birth order associations. Am. J. Epidemiol., 109: 66-70, 1979.

96. WHITELAW, A.G.L. The association of social class and sibling number with skinfold thickness in London schoolboys. Hum. Biol., 43: 414-420, 1971.

97. BRUCH, H. Eating Disorders. Basic Books, Inc., New York, 1973.

98. BULLEN, B.A., MONELLO, L.F., COHEN, H. and MAYER, J. Attitudes towards physical activity, food and family in obese and non-obese adolescent girls. Am. J. Clin. Nutr., 12: 1-11, 1963.

99. JELLIFFE, D.B. Infantile overnutrition. Brit. Med. J., 2: 546, 1973.

100. NEUMANN, C.G., and ALPAUGH, M. Birthweight doubling time: a fresh look. Pediatrics, 57: 469-473, 1976.

101. EID, E.E. Follow-up study of physical growth of children who had excessive weight gain in first six months of life. Brit. Med. J., 2: 74-76, 1970.

102. SWIET, M. de, FAYERS, P. and COOPER, L. Effect of feeding habit on weight in infancy. Lancet 1: 892-894, 1977.

103. FERRIS, A.G., BEAL, V.A., LAUS, M.J. and HOSMER, D.W. The effect of feeding on fat depostion in early infancy. Pediatrics, 64: 397-401, 1979.

104. DAVIES, D.P., GRAY, O.P., ELWOOD, P.C., HOPKINSON, C. and SMITH, S. Effect of solid foods on growth of bottle-fed infants in first three months of life. Brit. Med. J., 2: 7-8, 1977.

105. STUNKARD, A. and KAPLAN, D. Eating in public places: a review of reports of the direct observation of eating behavior. Int. J. Ob., 1: 89-101, 1977.

106. SCHACHTER, S. Obesity and eating. Science, 161: 751-756, 1978.

107. SCHACHTER, S. and RODIN, J. Obese Humans and Rats. Lawrence Erlbaum Associates, Potomac, MD, 1974.

108. A.C. NEILSEN COMPANY. 1980 Nielsen Report on Television. A.C. Neilsen Company, Northbrook, IL, 1980.

109. SHARAGA, S. Some effects of television advertising on children's nutrition attitude, nutrition knowledge, and eating habits. Unpublished Ph.D thesis. Cornell University, 1973.

110. DUSSERE, S.A. The effects of television advertising on children's eating habits. Unpublished M.Sc. thesis, University of Massachusetts, 1976.

111. BARCUS, E.F. Television in the Afternoon Hours. Action for Children's Television, Newtonville, ma 1975.

112. BARCUS, E.F. Weekend Commercial Children's Television. Action for Children's Television, Newtonville, MA 1975.

NUTRITION RESEARCH, Vol. 1, pp. 209-210, 1981
0271-5317/81/020209-02$02.00/0 Printed in the USA.
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REVIEWS OF BOOKS

Maternal Nutrition during Pregnancy and Lactation

Hugo Aebi and Roger Whitehead, Editors. Hans Huber Publishers, Bern. pp. 354, 1980.

The development of the fetus and the production of breast milk are of such fundamental importance for survival of the species that one might expect these twin processes to remain unaltered, even under the most adverse circumstances. However, objective data show that this is not invariable. Maternal malnutrition can retard fetal growth and development,as well as reduce the amount of milk produced. This volume records the proceedings of a Nestle Foundation workshop held in April, 1979. It addresses both basic and applied aspects of maternal nutrition during pregnancy and lactation. The results of dietary surveys and nutritional balance in different countries as varied as Sweden, U.S.A., the Gambia, New Guinea, Ethiopia and Britain are described. The methodology of nitrogen and mineral balance data is briefly reviewed. The importance of changes in physical activity producing alterations in nutritional needs is emphasized. The section on Basic Principles contains a pot-pourri of four papers with little common ground. Another section includes papers on the effects of inadequate nutrient intake during pregnancy and lactation. There is little critical analysis, however, of the possible reasons for the enormous variations in published results of several studies. Furthermore, the important effects of maternal undernutrition on the fetal development of immunity and postnatal susceptibility to infection are not mentioned. The final section analyses the results of some intervention studies. The article on the efficiency of criteria for selection of pregnant women for food supplementation asks very pertinent questions and provides tentative answers. It is important to judge the results of providing nutritional supplements during pregnancy, not only on the basis of birth weight, but also on the developmental and morbidity performance of the infant, and on the lactational performance of the mother. No study has addressed these questions.

The academic quality of the papers included in this compilation varies from fair to very good. There are some serious omissions and,as in many multiauthor books, a coherent interpretative analysis is sadly missing. In spite of these drawbacks, there are patches of useful information and the book would appeal to those who wish to familiarize themselves with the current state of the art in the subject of maternal nutrition.

Nutrition in Health and Disease

Myron Winick. John Wiley, New York. pp 261, 1980.

Of late, there has been a glut of textbooks on nutrition, each purporting to address itself to one or more segments of readership in the biomedical and health field. The need for a concise and clear exposition of the basic principles of human nutrition and their application to clinical medicine is recognized. This book by Winick, who has been at the forefront of nutrition education in the country, fills this gap to a large extent. The volume is divided into four logical sections. Part I discusses the role of dietary elements during pregnancy, infancy, adolescence and old age. Part II describes the

clinical and biochemical manifestations of nutrient deficiences. Part III addresses the question of excesses of nutrients and alcohol. Part IV summarizes dietary therapy in a few important disease states such as hypertension, renal disease and diabetes. The text is generally clear and very readable. However, there are some serious deficiences. There is obvious overlap in the chapters discussing excesses of calories on the one hand, and excesses of fat or carbohydrate on the other. There are some dogmatic statements and the topics of the cellular types of obesity and of nutrition and brain development reflect the author's own bias born out of his work. The subject of nutrition-immunity-infection is barely touched upon. In other sections, wrong statements reflect the author's unfamiliarity with the specific topic (for example, p.17.. maternal macrophages capable of producing antibody .....). The tabular material is useful and the printing is of good quality; the illustrations, however, are poorly reproduced. With these few limitations, the book is recommended and should prove useful for medical students and undergraduates in nutrition programs.

Advances in Modern Human Nutrition. Volume 1. Role of Nutrition in Obesity and Disease

Richard B. Tobin and Myron A. Mehlman, Editors. Pathotox Publishers, Park Forest South, Illinois, pp 365, 1980.

One would have liked to see a Preface justifying yet another Advances series. This first volume includes 13 essays on the role of nutrition in a variety of disease states such as obesity, aging, diabetes, cardiovascular disease and cancer; thus, the subtitle is misleading. The length and the quality of various contributions varies enormously; the majority are fair to good. The printing is excellent. One hopes for a broader coverage of nutritional problems in the next volume. It would be desirable to plan each volume either on one theme or alternatively to include a variety of topics.

NUTRITION RESEARCH, Vol. 1, pp. 211-212, 1981
0271-5317/81/020211-02$02.00/0 Printed in the USA.
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CONFERENCE SUMMARY

Third International Food Allergy Symposium

The meeting was organized by the American College of Allergists in Boston, October 18-21, 1980. A distinguished faculty reviewed all aspects of food intolerance. A summary of selected presentations follows: CHARLES D. MAY provided a historical perspective of the subject and discussed the principles of diagnosis and management. He cautioned against acceptance of study results other than those derived from double blind controlled trials of exclusion and challenge. JOHN F. SOOTHILL outlined the interactions between immunodeficiency and allergy. Human atopic allergy is related to a range of common minor defects of immunity function, such as transient IgA deficiency, defective yeast opsonization and low levels of complement component C_2. Prospective studies in newborns, fed exclusively on the breast and mutual exclusion of the defects in individual patients, provides supportive evidence that the immunologic deficits are causative. W. ALLAN WALKER described the phenomenon of antigen processing by the gastrointestinal tract. Food proteins, toxins and microorganisms can penetrate the mucosal barrier and enter the systemic circulation, causing clinical disease manifested by infection, allergy or autoimmunity. Both immunological and nonspecific barriers restrict the uptake of macromolecules. The role of the glycocalyx as an active metabolic compartment is being recognized. DEAN D. METCALFE described the role of the mast cell in gastrointestinal function and disease. The link between the appearance locally of invading organisms and subsequent mast cell degranulation is either IgE-antigen interaction or complement activation, resulting in the release of various chemical mediators of inflammation and vascular permeability. ANDERS DANNAEUS discussed the role of hereditary and developmental factors in food allergy. Cord serum IgE levels were predictive of subsequent development of atopic symptoms. At six months of age, the serum IgA levels were lower in the children of atopic mothers. High cord titers of IgG antibodies to milk proteins were found in children who remained asymptomatic, indicating a possible protective function. D.C. HEINER discussed the objective methods of diagnosis of cow's milk allergy and gluten-induced enteropathy. CLIFTON T. FURUKAWA described IgE specific suppressor cell dysfunction and overactive B cell function in some atopic patients. Antigen processing can be defective as a consequence of several mechanisms. SAMI L. BAHNA enumerated the respiratory manifestations of food allergy. JOHN W. GERRARD and C. COLLINS-WILLIAMS provided a clinical approach to the diagnosis and management of the food allergic patient. KJELL AAS discussed the advantages and limitations of skin prick tests and the radioallergosorbent test. He emphasized the need for highly reliable and purified allergen extracts for both techniques.

R.K. CHANDRA described the effects of nutritional deficiency on gastrointestinal immunity and its clinical consequences. Cell-mediated immunity is reduced as is secretory IgA response. The number of plasma cells and T lymphocytes is reduced. The significance of decreased intraepithelial lymphocytes is not clear. The permeability of the gut to macromolecules is increased. CHANDRA also discussed hypersensitivity to food additives such as pesticides, preservatives, flavouring agents, and dyes. Clinical manifestations include urticaria, angioedema, polymorphic skin rashes, abdominal pain and asthma. Diagnosis can be established by careful history, selected provocation tests and in vitro methods. JAMES M. SWANSON described the effects of food dyes on neurotransmitter accumulation and on behavior in rats and in hyperactive children. ROBERT

M. STROUD described the construction and use of a controlled environment unit for the diagnosis and management of difficult patients. Treatment of food allergy by dietary exclusion, cromolyn, and hyposensitization was summarized by a number of speakers.

The texts of various presentations and discussions are available as a series of tapes from the American College of Allergists.

NUTRITION RESEARCH, Vol. 1, pp. 213-225, 1981
0271-5317/81/030213-13$02.00/0 Printed in the USA.
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THE IMPACT OF SUPPLEMENTARY FEEDING AND HOME EDUCATION ON PHYSICAL GROWTH OF DISADVANTAGED CHILDREN

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ABSTRACT

Urban Colombian families with malnourished children were randomly assigned to treatment groups receiving either or both of a diet supplement and a home education program. The effectiveness of these interventions, both separately and interactively, in augmenting the physical growth of children during the first 36 months of life was assessed longitudinally. Children receiving the supplement were significantly taller and heavier than nonsupplemented controls after three months. Proportional weight for height over age was similar in all groups, while the supplement ameliorated the trend toward stunting. Home education moderately enhanced the supplementation effect, but alone did not improve growth. It is concluded that stunting without concomitant wasting may result from long-term deficiencies in calorie and protein intake and/or frequent disease episodes. Under such circumstances, measures of wasting (weight for height) would not be sensitive to the impact of long-term programs. Height for age would be a preferable outcome variable.

Key Words: Malnutrition, Child Development, Growth, Food: Supplemented

INTRODUCTION

Inadequate food supply is considered a major determinant of physical growth retardation among underprivileged children. If so, food supplementation and consequent improvement in dietary intake should prevent malnutrition and result in the recovery of those already malnourished. However, the effectiveness of food supplementation programs is not conclusive (1). Institutional treatment for the recuperation of protein-energy malnutrition has been successful (2-4), while the effectiveness of ambulatory supplementation of children at risk of malnutrition remains in doubt (1,5). What is more, most program evaluations focus on the impact of supplementation on the recuperation from protein/calorie malnutrition and not on its preventive effectiveness. Little is known, therefore, about the extent to which the incidence of

protein/calorie malnutrition can be diminished by supplementary feeding programs.

Beyond dietary intake, the etiology of protein/calorie malnutrition is embedded in the social environment. Illiteracy and poor educational backgrounds are associated with malnutrition, infection and inadequate feeding practices (6-8). Even within such environmental constraints, however, the mother may play an important role in promoting the child's health and nutrition (9). Maternal changes in attitude, motivation and ability to manage scarce household resources may be reflected in the child's health and development (10).

The purpose of the present study was to evaluate the effectiveness of supplementary feeding and maternal education (both separately and in combination) for the prevention of physical growth retardation among a sample of children at risk of malnutrition from the poor sectors of Bogota, Colombia. Previous reports have shown that supplementation improved the nutritional status of the study children's older siblings who were themselves below standard in weight for age (11), and also that supplementation improved the study children's weight and length gain (12). The present analysis comprises a more detailed examination of the interactive effects of supplementation and stimulation on growth and on the occurrence of wasting and stunting.

METHODS

These data were collected as part of a longitudinal study of the benefits of nutritional supplementation and social stimulation for child development. 443 children at risk of malnutrition and their families were studied in a prospective research project conducted in Bogota, Colombia. A description of the overall design and objectives has been published (13). Only those aspects of the study pertinent to current analysis are described below.

Subject families were selected from several adjacent poor neighborhoods (barrios) in Bogota. A survey was first conducted of all families in the area in order to identify those in which the mother was in the first or second trimester of pregnancy. A subset of these families which had children with substandard weight was identified as potential subjects for the study. Their expected offspring were considered at risk of malnutrition. Those families who elected to participate in a uniform health program, consisting of free obstetrical and pediatric care, were enrolled in the study and randomly assigned to experimental groups thereby allowing a controlled evaluation of the intervention.

The interventions consisted of a) a food supplementation program for all members of the family, and b) a home education program designed to enhance early stimulation of the subject children. In combination, these two interventions comprised a 2 x 2 experimental design with a control group, two groups with a single intervention, and one group that received both treatments.

Food supplements consisted of powdered skim milk, cooking oil, and enriched bread given in weekly allotments for the entire household. At least 623 calories and 30 grams of protein were provided for each family member. Additional portions were provided for pregnant mothers and appropriate supplements for infants beginning at three months of life. The supplements were sufficient to meet a substantial proportion of the recommended dietary allowances of each household member.

The stimulation program was designed to heighten the responsiveness of mothers and other caretakers to the child in order to strengthen reciprocal

interaction between them. Efforts were also made to enrich the physical environment of the children by providing simple toys constructed from available materials. The program, which was adapted from the Infant Educational Curriculum of the High/Scope Foundation, stressed awareness and understanding of the child's behavior as a means to promoting social interaction and stimulation. Mothers and other caretakers were visited at home twice a week by a trained visitor (14). Health and nutritional education were not planned components of the home visits.

Anthropometric measures of the study children were taken within 12 hours of birth and at 2, 3, 4, 6, 7, 9, 12, 18, 24, and 36 months of age. These measures included weight and supine length, head circumference, arm circumference, skinfold thickness, and chest circumference. Measurements were taken with standardized techniques by trained observers.

The results presented here pertain to weight and length measures and to several secondary indices derived from them. Growth increments were derived for each child by subtracting birth weight and length from weight and length at subsequent ages. Weight and length as percentages of age standards were calculated by dividing the child's weight and length by the 50th percentile standard for the appropriate age and sex (15). Proportional weight for length was calculated by dividing the child's weight by the corresponding standard weight for his/her length.[1]

Analyses of variance by stimulation, supplementation, and sex of the child were carried out on the several growth measures at all measurement points. In addition, the effect of the interventions on the distribution of subjects into categories of normal and subnormal nutritional status was examined utilizing commonly used classifications (16).

RESULTS

Mean weight and length by sex and experimental groups are shown in Tables I and II. Supplemented children exhibited greater height and weight than nonsupplemented subjects and boys tended to be larger than girls. The differences occurred in the expected directions and they increased with age to 36 months. Tables III and IV give the results of three-way analyses of variance on weight and length increments from birth to the different age points. Incremental scores were used in this analysis to reduce the variance attributable to prenatal factors. Supplementation and sex effects predominate: those of the former increasing with age and those of the latter diminishing. Differences due to stimulation were marginal and emerged late: simple stimulation effects were evident between 7 and 12 months and there were significant supplementation by stimulation interaction effects after 12 months. Children who received both treatments gradually emerged as the tallest and heaviest among the groups as can be seen in Tables I and II.

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[1] Weight for length was not calculated at ages under six months because some children in the sample fell below 49 centimeters in length which was the minimum reported with WHO reference standards. Attempts to use standards from other reference populations gave ambiguous results.

TABLE I.
Weight: Experimental Group Averages (kg)

	n	Age (Months) Birth	2	3	4	6	7	9	12	18	24	36
Control	70	2.95	4.35	4.96	5.41	6.23	6.52	7.18	7.98	9.08	10.05	11.86
Stimulation	38	2.88	4.33	4.93	5.46	6.27	6.61	7.17	7.98	8.87	9.73	11.57
Supplementation	69	2.99	4.50	5.07	5.66	6.55	6.92	7.54	8.35	9.46	10.57	12.34
Supplementation & Stimulation	46	2.89	4.49	5.18	5.73	6.63	7.05	7.68	8.63	9.69	10.76	12.70
All Boys	112	3.00	4.62	5.29	5.85	6.73	7.11	7.71	8.49	9.55	10.58	12.42
All Girls	109	2.90	4.24	4.79	5.29	6.14	6.48	7.08	7.84	8.93	9.93	11.79

TABLE II.
Supine Length: Experimental Group Averages (Cm)

	Age (Months) Birth	2	3	4	6	7	9	12	18	24	36
Control	47.7	54.1	56.8	58.8	61.9	63.0	65.2	68.3	73.5	77.9	85.4
Stimulation	47.1	53.8	56.4	58.4	61.3	62.7	65.1	68.2	73.2	77.7	84.7
Supplementation	47.7	54.6	57.2	59.5	62.7	64.0	66.4	69.7	75.1	79.9	87.5
Supplementation & Stimulation	47.5	54.2	57.1	59.5	62.8	64.2	66.5	70.3	75.9	81.1	88.8
All Boys	48.0	54.8	57.6	59.9	63.2	64.5	66.8	70.0	75.2	79.7	87.3
All Girls	47.3	53.8	56.2	58.3	61.5	62.7	65.0	68.2	73.6	78.3	86.0

When the growth measures were examined relative to the WHO standards (15), the trends reported above were somewhat altered. Sex differences were no longer significant suggesting the differences between males and females in the sample were similar to those found in the reference populations. The intervention effects, on the other hand, became more pronounced (Figures 1 and 2). The children in the control group, who presumably represented the usual growth pattern of children in this segment of the Bogota population, were born short and underweight relative to the standards. Between birth and 36 months of age their mean height decreased from 95 percent to 82 percent of the standards. The decrease in weight relative to the standard was precipitous until the age of nine months after which their weight disadvantage appeared to become stable. The decline in length in relation to standards was more gradual and prolonged. The supplemented groups followed a similar growth pattern but their losses in relation to the standards were not as pronounced as those of the controls. Their length disadvantage became stable at about 93 percent. Finally, those children who received only the stimulation intervention did not differ in the growth pattern from the control group, although stimulation did enhance growth when it was combined with food supplementation. The children who received both interventions were significantly taller after nine months of age than those who received only the supplement ($t=1.8$, $p<.05$).

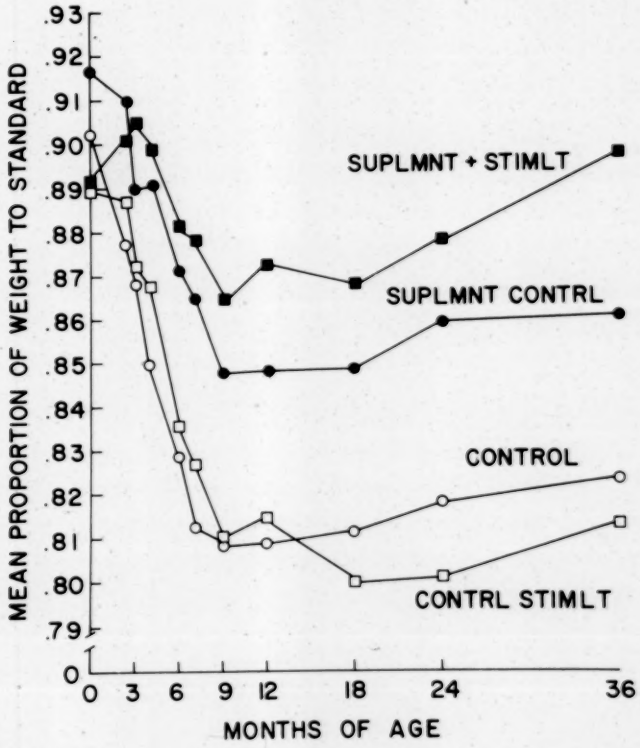

FIG. 1
Weight Relative to Standards by Experimental Group

The interventions had related effects on the distribution of normal and malnourished children when nutrition status was categorized according to usual anthropometric criteria (16). Differences betweeen supplemented and control groups at birth were small and significant only for boys (17). Thereafter, the children in all groups began to show deficits in weight for age and length for age. The deficits among those supplemented were not as great as those in the controls. By 36 months of age, the proportion of normal and malnourished children differed clearly among experimental groups (Table V). Between 20 and 24 percent of the nonsupplemented children were categorized as moderately malnourished, falling below 74 percent of weight for age. Only 8.7 percent of the supplemented subjects were moderately malnourished and only 2.2 percent of those who received both supplementation and stimulation fell in that category. Almost half of the subjects who received both interventions were normal in nutritional status by 36 months of age.

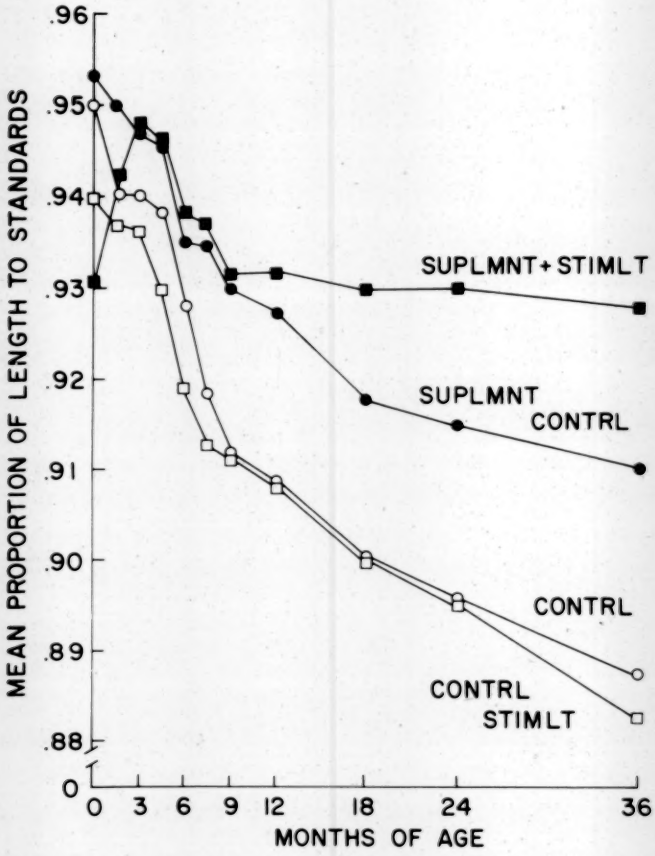

FIG. 2
Length Relative to Standards by Experimental Group

TABLE III.
F-Values for Analysis of Variance on Weight Increments from Birth

Contrasts	Age (Months) 2	3	4	6	7	9	12	18	24	36
Supplementation	4.28a	4.13a	7.90b	10.79c	13.62c	11.65c	14.63c	20.17c	24.73c	21.06c
Stimulation	.62	1.80	2.49	2.54	3.90a	2.29	3.10a	.66	.10	.78
Sex	31.52c	37.83c	43.83c	37.89c	35.37c	30.94c	26.29c	13.74c	12.28c	11.01c
Supplementation x Stimulation	.03	1.19	0	.13	.07	.70	1.71	3.46a	2.99a	3.63a
Supplementation x Sex	.03	.11	.07	.03	.33	.48	.60	.28	.34	1.18
Stimulation x Sex	1.16	.70	2.64a	1.67	.91	.04	.21	.14	.11	.32

TABLE IV.
F-Values for Analysis of Variance on Length Increments from Birth

Contrasts	Age (Months) 2	3	4	6	7	9	12	18	24	36
Supplementation	4.40a	3.95a	7.90b	17.80c	18.13c	14.47c	27.86c	30.74c	33.48c	33.96c
Stimulation	.03	.73	2.49	.21	1.07	2.00	3.38a	2.32	4.38a	1.99
Sex	2.50	14.40c	43.83c	21.17c	21.70c	19.78c	16.37c	4.58a	2.04	1.60
Supplementation x Stimulation	2.68a	0	0	.86	.28	.01	.89	1.98	1.40	2.14
Supplementation x Sex	2.75a	.74	.07	.01	.35	.42	2.64a	1.94	.86	1.03
Stimulation x Sex	.34	.99	2.64a	3.03a	2.68a	.91	1.43	.67	.25	.54

a $p<.05$
b $p<.01$
c $p<.001$

TABLE V.
Distribution of Children at 36 Months According to Categories of Nutritional Status (Gomez Classification) by Experimental Group.*

Nutritional Status Weight/Age	Control	Stimulated	Supplemented	Supplemented & Stimulated
Normal (above 90%)	21.4	18.4	27.5	45.6
Malnutrition:				
Mild (75%-90%)	58.6	57.9	63.8	52.2
Moderate (60%-74%)	20.0	23.7	8.7	2.2

* Numbers are percentage of cases within the corresponding experimental group.

The intervention effects on stunting (18) were similar to those on weight for age (Table VI). Few of the supplemented children, and none of those receiving both interventions, fell below 85 percent of length at 36 months of age. In contrast, 18.7 percent of the control and 26.3 percent of the children who received only the stimulation were stunted to that degree. At the upper end of the distribution, 12 percent of the supplemented, and 20 percent of the supplemented and stimulated children, were above 95 percent in length for age. Only one-third as many subjects in the nonsupplemented groups were in that range at 36 months of age.

TABLE VI
Distribution of Children at 36 Months According to Categories of Length for Age by Experimental Group.*

Percent Length for Age	Control	Stimulated	Supplemented	Supplemented & Stimulated
Above 95	7.1	5.3	11.6	19.6
90-95	37.1	34.2	49.3	60.8
85-90	37.1	34.2	33.3	19.6
Below 85	18.7	26.3	5.8	0.

* Numbers are percentage of cases within the corresponding experimental group.

Wasting, as indicated anthropometrically by subnormal weight for length, was not prevalent in any of the groups (Figure 3). The mean values for all the experimental groups were somewhat above the standards in weight for length at six months.[2] The means decreased to slightly below the standards at 18 months of age and then returned to 100 percent by 36 months of age.

[2] The finding that the Bogota children under one year of age are chubbier than the reference population appears surprising but is empirically correct. At these same lengths, children in the reference population would tend to be younger, and it can be speculated that they would not have had as much time as the Bogota children to deposit adipose tissue.

Figure 3 also shows that neither supplementation nor stimulation resulted in significant group differences in weight for length. Supplemented children, although heavier and longer than the nonsupplemented controls, maintained similar ratios in weight for length, suggesting therefore, that the main effect of supplementation was on linear growth.

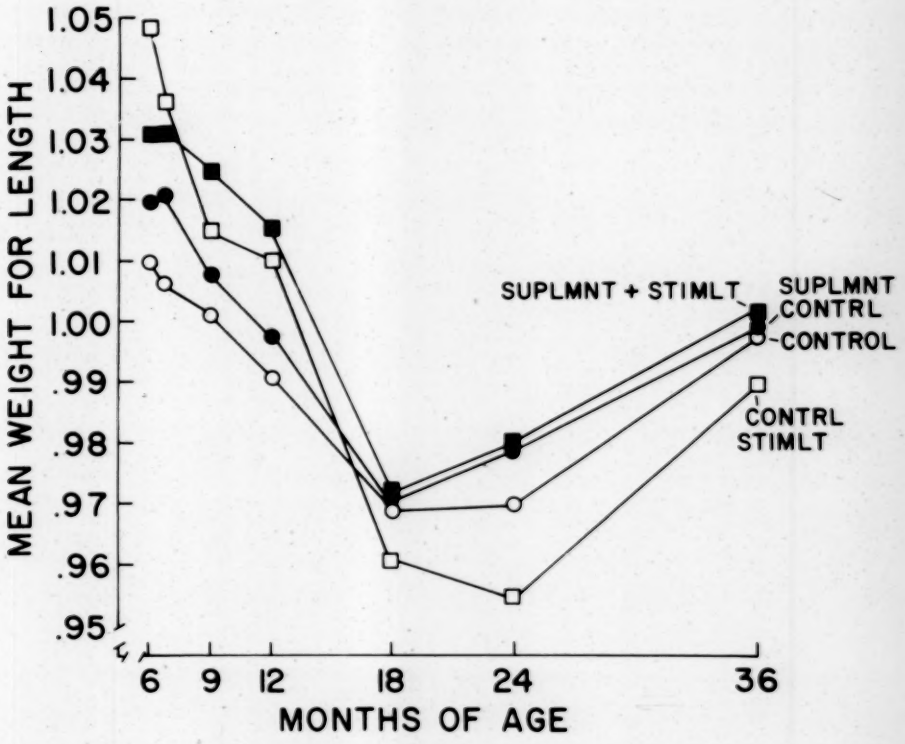

FIG. 3
Weight Relative to Length Standards by Experimental Group

DISCUSSION

It is evident from the results reported above that the supplementation program had a significant effect on physical growth. The differences in weight and length between supplemented and nonsupplemented groups become increasingly significant with time. The beneficial effects of supplementation were enhanced by the stimulation program which encouraged greater responsiveness of the mothers to their offspring. The home visiting program by itself did not have an impact on physical growth.

The interaction effect of stimulation and supplementation, although barely significant statistically, is of interest considering that the home education program did not deliberately include a nutrition education component. The emphasis was on orienting the mother's attention toward the child's psychosocial development. It is conceivable that maternal awareness of the child's needs and increased positive interaction between caretaker and subject children accounted for the interactive effects of the stimulation and

supplementation programs (10). Hypothetical pathways for such an interaction could be: a) through reduced gastrointestinal morbidity; or b) a net increase in calorie and protein intake.

The supplementation effects reported above are similar but more pronounced than those of supplementation programs elsewhere (19-22). As a preventive measure, the supplementation program considerably reduced the incidence of moderate malnutrition. The familiar nature of the foods in the supplementation program, the fact that rations were provided for every member of the family, and the high risk of malnutrition to which the study children were exposed (as evidenced by the physical growth retardation in their older siblings) may explain the effectiveness of the program.

The interventions had the effect of ameliorating a deteriorating growth trend among the study children relative to international growth standards: even those children who received the supplement did not attain the levels of weight or height expected for their ages. What the supplementation did accomplish was to reduce the cumulative deficit. That these children did not "catch-up" to the standards is apparent, though not fully explained here. It should be noted, however, that they were already shorter and lighter than the norms at birth and so their growth potential may have been constrained by maternal factors or intrauterine events.

At birth the study children were two centimeters shorter than the standards, independent of supplementation which increased birth weight marginally, but not length. Given the short stature of the mothers (mean height 149.7 $\pm$ 5.4 cm, range 132.8/165 cm) it may be hypothesized that maternal body build imposed limitations to fetal growth independent of maternal diet (23). In that case, supplementation during pregnancy and thereafter, would affect only the height deficit attributable to nutritional deprivation.

A third point that deserves emphasis is that the study children as a whole were not substandard in weight for length and supplementation had no significant effect on that parameter. In other words, the malnourished children in the sample were stunted and their weight was a function of their stature. Supplementation affected linear growth, and differences in weight for age were accounted for by the supplementation effect on height. Stunting is the usual anthropometric indicator of long-term protein/calorie malnutrition (24). Teleologically it can be proposed that children's growth decelerates until a rate is attained which can be sustained by the available diet. In the findings presented above, it is evident that children's weight gain approached that of the standards at about 9 months of age, whether they were supplemented or not. By that time, the supplemented children, because of their relatively faster linear growth, had a smaller weight deficit than the controls.

The relatively low proportion of wasting (a maximum of 16 percent at 24 months among the nonsupplemented) is a common finding in Latin American nutrition studies as opposed to much higher figures reported in certain areas in Asia and Africa (19,24,25). Wasting often results from episodes of famine or epidemic waves of serious communicable child diseases such as measles and whooping cough, currently not common in Latin America. Stunting without concomitant wasting may result from long-term deficiencies in calories and protein intake and/or frequent disease episodes. The results reported above clearly suggest that under these circumstances measures of wasting, such as weight for height, cannot be expected to be sensitive indicators of the nutrition impact of long-term programs. Height for age (or even weight for age which also reflects height) would be preferable to weight for height as an outcome variable.

Linear growth retardation in itself may not be a significant function impediment for those who survive early malnourishment (26). We have reported elsewhere that the interventions enhanced children's behavior and cognitive

development (27), but whether the effects occurred through the impact of supplementation on physical growth, or were independent of it, remains to be established. The physical and developmental implications of small stature have not been fully unravelled, i.e. to what extent is the adaptation of body size to prevailing environmental conditions a physiological mechanism that forestalls the more serious consequences of chronic malnutrition (28).

ACKNOWLEDGEMENT

Supported in part by the Colombian Institute of Family Welfare, NICHHD Grant No. HD06774, The Ford Foundation Grant No. 740-0348, and the Fund for Research and Teaching, Department of Nutrition, Harvard School of Public Health.

REFERENCES

1. BEATON, G.H. and GHASSEMI, H. Supplementary feeding programs for young children in developing countries. Report for UNICEF and the ACC Sub-Committee on Nutrition of the United Nations. New York, 1979.

2. BEAUDRY-DARISME, M. and LATHAM, M. Nutrition rehabilitation centers - An evaluation of their performance. J. Trop. Pediatr. Environ. Child Health, 19: 299-332, 1973.

3. BEGHIN, I.D. and VITERI, F.E. Nutritional rehabilitation centers: An evaluation of their performance. J. Trop. Pediatr. Environ. Child Health, Monograph No. 31, 19: 403-416, 1973.

4. BROOKE, O.G. and WHEELER, E.I. High energy feeding in protein-energy malnutrition. Arch. Dis. Child, 57: 968-971, 1976.

5. MARTORELL, R., LECHTIG, A., YARBROUGH, C., DELGADO, H., and KLEIN, R. Protein-calorie supplementation and postnatal physical growth: A review of findings from developing countries. Arch. Latinamer. Nutr., 26: 115-128, 1976.

6. CHRISTIANSEN, N., VUORI, L., MORA, J.O., and WAGNER, M. Social environment as it relates to malnutrition and mental development. In: Early malnutrition and mental development. J. Cravioto, L. Hambraeus, and B. Vahlquist(eds). Almqvist & Wiksell, Uppsala, 1974.

7. SANJUR, D., CRAVIOTO, J., and VAN VEEN, A.G. Infant nutrition and sociocultural influences in a village in Central Mexico. Trop. Geograph. Med., 22: 443-451, 1970.

8. WRAY, J. and AGUIRRE, A. Protein-calorie malnutrition in Candelaria, Colombia. I. Prevalence: social demographic causal factors. J. Trop. Pediatr., 15: 76-98, 1969.

9. RAWSON, I. and BERGGREN, G. Family structure, child location and nutritional disease in rural Haiti. J. Trop. Pediatr. Environ. Child Health, 19: 288-298, 1973.

10. SUPER, C., CLEMENT, J., VUORI, L., CHRISTIANSEN, N., MORA, J.O., and HERRERA, M.G. Infant and caretaker behavior as mediators of nutritional and social intervention in the barrios of Bogota. In: Culture and early interaction. T. Fields(ed). Erlbaum, Hillsdale (in press).

11. MORA, J.O., HERRERA, M.G., and SUESCUN, J. The effect of food supplementation on the physical growth of undernourished preschool children (in preparation).

12. MORA, J.O., HERRERA, M.G., SUESCUN, J., de NAVARRO, L., and WAGNER, M. The effects of nutritional supplementation on physical growth of children at risk of malnutrition. Am. J. Clin. Nutr., (in press).

13. HERRERA, M.G., MORA, J.O., CHRISTIANSEN, N., ORTIZ,N., CLEMENT, J., VUORI, L., WABER, D., de PAREDES, B., AND WAGNER, M. Effects of nutritional supplementation and early education on physical development, In: Life-Span developmental psychology: Intervention. R.R. Turner and R.W. Reese (eds.). Academic Press, New York, 1980,pp. 149-184.

14. CLEMENT, J., and FLOREZ, A. An Environmental stimulation program for children at high environmental risk in Bogota Colombia: A summary of the children's first year. Presented at the IV Congress of the International Association for the Scientific Study of Mental Deficiency, Washington, DC, August 22-27, 1976.

15. WORLD HEALTH ORGANIZATION. A growth chart for international use in maternal and child health care; Guidelines for primary health care personnel. World Health Organization Nonserial Publication, Geneva, 1978.

16. GOMEZ, F., RAMOS, G.R., FRENK, S., CRAVIOTO, J., CHAVEZ, R., and VASQUEZ, J. Mortality in second and third degree malnutrition. J. Trop. Pediatr. 2:77-83, 1956.

17. MORA, J.O., de PAREDES, B., WAGNER, M., de NAVARRO, L., SUESCUN, J., CHRISTIANSEN, N., and HERRERA, M.G. Nutritional supplementation and the outcome of pregnancy. I. Birth Weight. Am. J. Clin. Nutr. 26:563-566, 1979.

18. WATERLOW, J.C. Classification and definition of protein-energy malnutrition, In: Nutrition in Preventive Medicine. G.H. Beaton and J.M. Bengoa (eds.). World Health Organization Monograph Series No. 62, Geneva,1976, pp. 530-555.

19. MARTORELL, R., KLEIN, R., and DELGADO, H. Improved nutrition and its effects on anthropometric indicators of nutritional status. Nutr. Rept. Internat. 21:219-230, 1980.

20. GOPALAN, C., SWAMINATHAN, M.C., KRISHNA KUMARI, V.K., HANUMANTHA RAO, D., and VIJAYARAGHAVAN, K. Effect of calorie supplementation on growth of undernourished children. Am. J. Clin. Nutr. 26:563-566, 1973.

21. RAO, D.H., and NADAMUNI, N.A. Nutritional supplementation - Whom does it benefit most? Am. J. Clin. Nutr. 30:1612-1616, 1977.

22. EDOZIEN, J. C., SWITZER, B.R., and BIJAN, R.B. Medical evaluation of the special supplemental food program for women, infants and children Am. J. Clin. Nutr. 32:677-692, 1979.

23. THOMSON, A.M. Diet in pregnancy: Diet in relation to the course and the outcome of pregnancy. Br. J. Nutr. 13:509-525, 1959.

24. KELLER, W., DONOSO, G., and DEMAEYER, E.M. Anthropometry in nutritional surveillance: A review based on results of the WHO collaborative study on nutritional anthropometry. Nutr. Abstracts Rev. 46:591-609, 1976.

25. ANDERSON, M.A. Comparison of anthropometric measures of nutritional status in preschool children in five developing countries. Am. J. Clin. Nutr. 32:2339-2345, 1979.

26. MORA, J.O., CLEMENT, J., CHRISTIANSEN, N., SUESCUN, J., WAGNER, M ., and HERRERA M.G. Nutritional supplementation and the outcome of pregnancy. III. Perinatal and neonatal mortality. Nutr. Rept. Internat. 18:167-175, 1978.

27. WABER, D., CHRISTIANSEN, N., ORTIZ, N., CLEMENT, J., VUORI, L., MORA, J.O., REED, R., and HERRERA, M.G. Effects of nutritional supplementation and maternal stimulation on cognitive development of infants at risk of mild-to-moderate malnutrition, (in press).

28. MARTORELL, R., LECHTIG, A., YARBROUGH, C., DELGADO, H., AND KLEIN, R.E. Small stature in developing nations: Its causes and implications., In: Progress in human nutrition, S. Margen and R.A. Ogar (eds.). The Avi Publishing Company, Westport, 1978, pp. 143-156.

NUTRITION RESEARCH, Vol. 1, pp. 227-233, 1981
0271-5317/81/030227-07$02.00/0 Printed in the USA.
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COMPARATIVE TOLERANCE OF ADULTS OF DIFFERING ETHNIC BACKGROUNDS TO LACTOSE-FREE AND LACTOSE-CONTAINING DAIRY DRINK

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ABSTRACT

Milk intolerance secondary to lactose malabsorption was investigated in 99 healthy adults (18 to 46 years old) of differing ethnic backgrounds. A breath hydrogen test (BHT) following a 250 ml. oral dose of a 10% lactose solution identified 24 subjects as lactose malabsorbers. A lactose-free (LF) chocolate-flavored dairy drink and one containing 4.5% lactose (LC) were formulated to simulate low-fat chocolate milk. These were consumed in 240 ml. and 480 ml. volumes under double-blind conditions to determine milk intolerance. Only three of the lactose malabsorbers appeared intolerant to both 240 ml. and 480 ml. of the LC dairy drink. No statistically significant differences were found in the symptom prevalence reported by lactose malabsorbers (LM) and lactose absorbers (LA) after 240 ml. of LC and LF dairy drink. The difference in symptoms between the LM and LA was significant ($p < .002$) after 480 ml. of LC dairy drink and after the BHT ($p < .002$). The lactose-in-water administered for the BHT produced more symptoms in LM than did the equivalent amount of lactose in the dairy drink ($p < .04$). Symptoms reported by LA to the LC dairy drink indicated that gastrointestinal distress occurring after consumption of a lactose-containing dairy drink was not necessarily due to lactose malabsorption.

Milk intolerance Lactase deficiency Lactose malabsorption

INTRODUCTION

In 1972 the Protein Advisory Group (PAG) of the United Nations System clarified the terminology used for the subject of lactose malabsorption (1). 'Low lactase activity' was defined as "less than two units of lactase activity is equal to one μmole of lactose hydrolyzed per minute per gram wet mucosa." 'Lactose malabsorption' was defined as "reduced absorption of lactose, a consequence of low lactase activity, determined by lactose tolerance tests." 'Lactose intolerance' was likewise defined as the occurrence of "clinical signs (abdominal pain, flatulence, bloating, and diarrhea) following ingestion of lactose mixed in water in standard dose or less, in a person with proven lactose malabsorption." Lastly, milk intolerance was defined as the appearance of "clinical signs consistently following a few hours after ingestion of milk or milk-containing products in usual amounts in a person with proven lactose malabsorption."

Low lactase activity can be of congenital, secondary, or primary origin. The congenital form is an inborn error manifest at birth and persisting throughout life. Secondary low lactase activity is a result of inflammatory intestinal disease and is usually associated with a temporary decrease in all disaccharidases. Primary low lactase activity (also referred to as adult, isolated, or racial) describes a condition in which lactase is the only disaccharidase at low activity level. The majority of adults in the world who are not of northern

European descent, and many who are, have primary low lactase activity. They may or may not experience the characteristic symptoms of lactose intolerance, as defined by the PAG, when given a test quantity of milk, because the range of subjective and physiologic milk intolerance among lactose-intolerant subjects is extremely wide (2-5).

Some investigators have found that most lactose-intolerant persons can consume nutritionally valuable quantities of milk without adverse symptoms (4). On the other hand, when a double-blind procedure was used to determine symptoms directly attributable to the lactose in milk and not to other physiological factors, it appeared that many malabsorbers who reported symptoms in a hospital setting were reacting to lactose and thus limiting their milk consumption (6). Clearly, the way in which investigators monitor symptoms, or conduct population studies can produce varying results.

To illustrate the causal relationships between low lactase activity and symptoms of intolerance induced by lactose alone and lactose in milk, the responses of randomly selected young adults to a chocolate-flavored dairy drink with and without lactose were studied. The techniques were those used by Kwon et al. with adolescents, in which 240 ml. and 480 ml. volumes were administered randomly on a double-blind basis (7). Intestinal lactase activity levels were indirectly measured by use of a breath hydrogen test (BHT). The BHT has been shown to be the best indirect method for determining lactose-absorbing ability (8,9). It is least invasive and most sensitive for a relatively small test dose of lactose.

METHODS

Subjects: Ninety-nine healthy adults, 18 to 46 years old (mean 23.63 ± 5.3), 49 of them female, volunteered from the MIT community to participate in the study. The subjects described their ethnic origins as predominantly non-Jewish northern European, 64; Jewish, 14; non-Jewish southern European, 8; Oriental, 7; Asian Indian, 3; Black, 3; Middle Eastern, 1.

Breath hydrogen test: An initial breath sample was collected in the morning after a ten-hour fast. Each subject was then given 250 ml. of a 10% lactose-in-water solution, slightly flavored with lemon juice, and instructed to drink the solution within a few minutes. Additional breath samples were collected at 90, 120, 150, and 180 minutes after the initial sample. Between the fasting and 90-minute samples, the subjects were served a light breakfast consisting of sweet rolls, coffee, and tea, without milk or cream, and orange juice.

Breath samples were drawn via nasal cannula, using a 50 ml. disposable syringe adapted with a stopcock and plastic tubing for easy collection. Samples were immediately transferred to evacuated test tubes to await analysis by gas chromatography. A rise of 17 ppm above the fasting level was considered indicative of lactose malabsorption.

At the conclusion of the test, subjects were given a form on which to report all symptoms that occurred during the three-hour period (bloating, flatulence, cramps, diarrhea, or other) by checking (a) none; (b) mild; (c) moderate; or (d) severe. They were asked to specify the time of symptom onset, and in addition, to notify the investigator if symptoms worsened or if new ones occurred during the day of the test. The presence of one or more of these symptoms between one-half hour and 24 hours after ingestion of the solution was considered a positive response to lactose.

On the morning of the BHT, each subject completed a questionnaire on age, ethnic heritage, beliefs about milk, use of milk and dairy products, discomfort experienced after consuming milk or dairy products, chronic digestive and medical problems, and dietary habits that might affect the study.

Dairy drink lactose tolerance test: All subjects were tested on a double-blind basis with 240 ml. and 480 ml. doses of lactose-free (LF) and lactose-containing (LC) chocolate-flavored dairy drink as previously used by Haverberg (10), and described in Table 1. Most doses of the dairy drink were consumed

between 11 A.M. and 2 P.M., and subjects were asked only to refrain from drinking milk or eating ice cream for four hours afterward. Symptomatology questionnaires were distributed to subjects each day after the drink was consumed. The same symptom form and procedure for its completion used with the BHT were employed. One or more relevant symptoms occurring between one and 24 hours indicated a positive response to the dairy drink for that test day.

TABLE 1

Composition of Chocolate-Flavored Dairy Drink[a]

Ingredient	Lactose-Free Dairy Drink	Lactose-Containing Dairy Drink
	% Composition	
Coconut fat[b]	1.00	1.00
Sodium and calcium caseinate[c]	3.00	3.00
24 DE corn sugar[d]	5.44	----
Cane sugar[d]	8.00	7.79
42 DE corn sugar[d]	----	1.14
Salt (NaCl)[e]	0.18	0.18
Cocoa[f]	1.20	1.20
Carrageenan[g]	0.05	0.05
Polysorbate, mono and diglycerides[h]	0.10	0.10
Water	81.03	81.04
Lactose[i]	----	4.50

[a]Manufactured and supplied by H.P. Hood, Inc., Boston, Massachusetts. [b]Used as a substitute for milk fat, because milk fat, in the form of cream, would introduce lactose in the lactose-free product. [c]Provides the same amount of protein normally found in "low-fat chocolate milk" but has a lower protein efficiency ratio than the equivalent amount of total protein or whole-milk protein found in milk. [d]Contributes sweetness and extra solids to enhance taste and texture. [e]Added to enhance flavor. [f]18% Dutch process cocoa. [g]Provides body and suspends cocoa fiber. [h]Added as emulsifiers for fat suspension. [i]Added to provide the equivalent amount of lactose normally found in "low-fat chocolate milk."

RESULTS

Breath hydrogen test. According to the criteria mentioned, 24 subjects were identified as lactose malabsorbers. Of the LM, 62.5 percent and of the LA, 30.7 percent reported symptoms, representing a significant difference between the two groups ($p < .002$). Results also showed that there was a statistical significance ($p < .002$) between LA and LM relating the lactose-absorbing status to the ethnic heritage of the subjects. Among persons of predominantly non-Jewish northern European origin, approximately 13 percent were lactose malabsorbers compared to 41 percent for all the groups combined.

Dairy drink lactose tolerance test. Table 2 gives the various responses of the subjects to the dairy drink lactose tolerance test. Twenty-nine (38.6 percent) of the LA and 13 (54.2 percent) of the LM reported symptoms on at least one test day. The largest response category was that of individuals judged tolerant to LC dairy drink in volumes of 480 ml. or less. Of the 85

individuals in this group, 69 (81 percent) were LA and 16 (19 percent) were LM. No statistical differences were found in the symptom prevalence reported by LA and LM after 240 ml. of LC and LF dairy drink, but the difference was significant ($p < .002$) after 480 ml. of LC dairy drink. Three individuals describing symptoms after both 240 ml. and 480 ml. of the LC dairy drink were considered lactose-intolerant. Three absorbers (4 percent of LA) and three malabsorbers (12.5 percent of LM) reported symptoms only after 480 ml. of the LC dairy drink.

TABLE 2

Tolerance Classification of Subjects by Response to Graded Amounts of Lactose-Free (LF) and Lactose-Containing (LC) Dairy Drink

Tolerance Classification		Symptom Response				
No. of Subjects		240 ml.	240 ml.	480 ml.	480 ml.	
LA[a]	LM[b]	LF	LC	LF	LC	
46	11	-[c]	-	-	-	Tolerant to 480 ml.
8	1	X[d]	-	-	-	of LC drink
2	0	X	X	-	-	
1	0	X	X	X	-	
1	0	X	X	X	X	
4	2	-	X	-	-	
0	1	-	X	X	-	
2	1	-	X	X	X	
1	0	-	-	X	-	
1	0	-	-	X	X	
3	0	X	-	X	-	
0	0	X	-	X	X	
3	3	-	-	-	X	Intolerant to 480 ml. of LC drink
0	3	-	X	-	X	Intolerant to 240 ml. and 480 ml. of LC drink
1	1	X	X	-	X	Intolerant to 480 ml.
2	1	X	-	-	X	of LC drink. Relation to lactose doubtful.
Total						
75	24					

LA[a] = lactose absorber
LM[b] = lactose malabsorber
-[c] = symptoms absent
X[d] = symptoms present

On the assumption that the symptoms in the LA were not caused by the lactose content of the LC dairy drink, the percentage of malabsorbing individuals intolerant to the lactose in 240 ml. of LC dairy drink was 12.5 percent, and to 480 ml. was 25 percent. The 5.3 percent of LA reporting symptoms after only 240 ml., and 4 percent after 480 ml. of LC dairy drink provide an estimate of the possible number of false positives. Intolerance to 480 ml. of LC dairy drink in five other subjects (3 LA and 2 LM) was considered doubtful because they reacted to 240 ml. of LF as well as to one or both levels of LC dairy drink.

A scoring method was used to quantify symptoms reported by each subject for dairy drink consumption and the BHT. The four symptoms (bloating, flatulence, cramps, diarrhea) indicative of lactose intolerance were rated according to severity by the subjects themselves. The scoring was: none = 0; mild (noticeable but not bothersome) = 1; moderate (uncomfortable) = 2; and severe (definitely painful, difficult to carry on daily routine) = 3. Thus, a score for a single test could range from 0 to 12. The mean scores for the LF dairy drink at 240 ml. and 480 ml. volumes were: .24 and .26, and for the LC dairy drink at 240 ml. and 480 ml. volumes, .43 and .42, respectively. The BHT mean score was 1.01. The BHT produced more symptoms in the LM than approximately the same amount of lactose in the dairy drink (pc .04).

Questionnaires completed by subjects reported that most liked both plain and chocolate milk, and that they had learned as children that milk was a good nutritional source. Subjects normally used milk in three ways: as a drink, on cereal, in tea or coffee, and in cooking. The mean amount of milk drunk per week per person was 2.84 liters (range 0 to 30.28 liters). The mean amount of milk reported to be consumed at one time was 229 ml. Subjects reported that they ate two of the following dairy products at least once per week: buttermilk, cottage cheese, American cheese, ice cream, and yogurt.

Subjects were asked to rate chronic symptoms (flatulence, diarrhea, constipation, and abdominal pain) as mild, moderate, or severe. Using the same scoring scheme described earlier, the mean score for digestive problems was 1.4. Regarding discomfort caused by milk ingestion, five said they always had it, 29 sometimes had symptoms, and 64 never experienced it. When asked about discomfort from other dairy products, three said always, 20 sometimes, and 74 never. A significant difference was found in the prevalence of discomfort caused by milk reported by LA and LM ($p < .01$). Other questions about medical problems and dietary habits on the questionnaire revealed no significant differences in responses between the LA and LM groups.

DISCUSSION

Presence and severity of symptoms are partially related to growth in the colon of bacterial flora with the metabolic capacity to digest lactose. In theory, lack of such flora can produce false negatives with the BHT. Conversely, an abundance of the proper microorganisms acting in a sufficiently rapid manner might utilize the lactose in the colon of a malabsorber and reduce or prevent gastrointestinal symptoms. Perhaps this explains one subject with a high fasting-level H_2 output who had a rise of 55 ppm of hydrogen but remained symptom-free throughout the BHT and dairy drink tests.

Another subject stated that, after abstinence from milk for a month or more, reaction to consuming only 240 ml. of milk could be quite severe, but he was able to build up his tolerance by a gradual increase in volume. It is notable that this subject reported no symptoms to either LC or LF dairy drink, but experienced flatulence, bloating, and diarrhea with the BHT and had a rise of 18 ppm of H_2.

Welsh and Hall (11), using various solutions, tested LA and LM subjects for gastric emptying time and found that chocolate milk, with a higher osmolarity than plain milk, emptied more slowly and that emptying time for lactose malab-

sorbers was significantly lower. Some symptoms of lactose malabsorption are related to the rate at which lactose is delivered to the intestine. The use of a chocolate dairy drink might contribute to the failure of two lactose malabsorbers to report symptoms despite a supposed reaction to ordinary milk. The significant difference in the prevalence of symptoms reported by LA and LM for 480 ml. of the LC dairy drink suggests that there was sufficient lactose present to override a possible symptom-suppressing effect of the chocolate. Whether there would have been a greater distinction in occurrence of symptoms between the LA and LM groups with plain milk is unknown.

Because subjects reported symptoms according to their individual points of view, it is difficult to be sure of the accuracy of the symptom data. The best standards for comparison were the definitions given to the symptom ratings, although the manner in which an investigator requests a report of symptoms, even in questionnaire form, may influence the response. The data showed that gastrointestinal distress reported after a lactose-containing dairy drink is not necessarily caused by lactose malabsorption.

In practice, lactose-intolerant individuals must limit their consumption of milk and other lactose-containing dairy products to the point of their individual tolerance levels. Some may have to abstain from milk consumption but may be able to tolerate a few ounces of ice cream. Others may be able to avoid unpleasant symptoms, and still consume milk, by eating sufficient quantities of solid food to slow G.I. transit time and hence, the presentation of lactose to the small intestine. When it is established that their gastrointestinal distress results from low intestinal lactase activity, individuals with this problem soon learn their own dietary limitations.

REFERENCES

1. PROTEIN ADVISORY GROUP OF THE UNITED NATIONS (PAG). Ad Hoc Working Group on Milk Intolerance -- Nutritional Implications. PAG Bulletin 2: 7, 1972.

2. BAYLESS, T.M. Recognition of lactose intolerance. Hospital Practice 11: 97, 1976.

3. SIMOONS, F.J., JOHNSON, J.D., AND KRETCHMER, N. Perspective on milk-drinking and malabsorption of lactose. Pediatrics 59: 98, 1977.

4. STEPHENSON, L.S. AND LATHAM, M.C. Lactose intolerance and milk consumption: the relation of tolerance test to symptoms. Am. J. Clin. Nutr. 27: 296, 1974.

5. GUDMAND-HOYER, E. AND SIMONY, K. Individual sensitivity to lactose in lactose malabsorption. Am. J. Digest. Dis. 22: 177, 1977.

6. BAYLESS, T.M., ROTHFELD, B., MASSA, C., WISE, L., PAIGE, D., AND BEDINE, M. Lactose and milk intolerance: clinical implications. New Eng. J. Med. 292: 1156, 1975.

7. KWON, P.H., RORICK, M.H., AND SCRIMSHAW, N.S. Comparative tolerance of adolescents of differing ethnic backgrounds to lactose-containing and lactose-free diary drinks II. Improvement of a double-blind test. Am. J. Clin. Nutr. 33: 22, 1980.

8. BOND, J.H. AND LEVETT, M.D. Quantitative measurement of lactose absorption. Gastroenterology 70: 1058, 1976.

9. NEWCOMER, A.D., THOMAS, P.J., McGILL, D.B., AND HOFMANN, A.F. Comparison of methods to detect lactase deficiency. Gastroenterology 66: 754, 1974.

10. HAVERBERG, L., KWON, P.H., AND SCRIMSHAW, N.S. Comparative tolerance of adolescents of differing ethnic backgrounds to lactose-free and lactose-containing dairy drinks I. Initial experience with a double-blind procedure. Am. J. Clin. Nutr. 33: 17, 1980.

11. WELSH, J.D. AND HALL, W.H. Gastric emptying of lactose and milk in subjects with lactose malabsorption. Am. J. Digest. Dis. 22: 1060, 1977.

NUTRITION RESEARCH, Vol. 1, pp. 235-241, 1981
0271-5317/81/030235-07$02.00/0 Printed in the USA.
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SELENIUM CONTENT OF SELECTED HOSPITAL DIETS

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ABSTRACT

Selenium has been recently recognized as an essential trace element for humans with a daily intake of 50-200 µg suggested. Typical hospital meals were collected during a seven-day period at breakfast, lunch and dinner for regular meat-based, ovo-lacto-vegetarian, and low calorie-low protein renal diets. The selenium content of each diet was determined using neutron activation analysis. Total selenium content of the daily diets averaged 85.5 µg for regular, 118 µg for vegetarian and 54.6 µg for renal diets. The actual intakes of selenium by the subjects would be less because frequently all of the food was not consumed. Thus, the mean selenium level fell within the recently suggested range of 50-200 µg per day. However, the low-calorie-low protein renal diet was barely adequate and the regular diet was near the lower limit of the recommended range estimated as safe and adequate.

Key Words: Selenium, Hospital Diets, Trace Elements, Recommended Daily Intakes.

INTRODUCTION

Selenium, a trace element, is accepted as essential for humans. However, there are more data regarding the essentiality of selenium for animals than for man (1-6). Nevertheless, an "estimated safe and adequate" daily intake of selenium in man has recently been set at 50-200 µg per day by the Food and Nutrition Board (7). Average total selenium content of the body was estimated as 14.6 mg (13-20 mg); with approximately 100-150 µg per day eliminated via urine, feces, skin and respired air (8).

In general, the best sources of selenium in foods consumed by humans are meat, seafood, and grains. Milk, milk products, fruits and vegetables are poorer sources (9, 10, 11). Morris and Levander (20) indicated that a diet well balanced in other nutrients probably is also nutritionally adequate in selenium; however, they cautioned that the possible effects of processing, cooking, bioavailability and geographic variation might affect the adequacy.

The objectives of this study were: to determine by neutron activation analysis the selenium content of the daily diets served at a hospital and to compare the daily selenium content of these diets with the recently established recommended daily intake.

METHODS

Meal collection: We examined regular meat-based diets, lacto-ovo vegetarian, and low protein-low caloric renal diets served at the VA Medical Center,

Washington, D.C. Trays of these three diets were collected for breakfast, lunch and dinner meals for 7 consecutive days. Zinc in the diets was determined by atomic absorption spectrophotometry (12), and protein and calories were calculated from tables (13). The content of zinc, protein and calories in these diets was reported earlier (14). Samples of some meals were insufficient for selenium analysis. The actual number of meals analyzed per type of diet ranged from 3 to 6 and appears in Table 1.

TABLE 1

Selenium Content of Various Diets

Meal	Selenium per meal (μg)	Selenium per day (μg)
Regular (Meat-Based)		
Breakfast	28.4 ± 9.6 (3)*	
Lunch	32.5 ± 8.8 (5)	85.5
Dinner	24.7 ± 7.1 (3)	
Range for individual meals	(12.1 - 65.2)	
Vegetarian (lacto-ovo)		
Breakfast	62.5 ± 13.2 (3)*	
Lunch	31.1 ± 9.0 (3)	118.1
Dinner	24.6 ± 5.1 (5)	
Range for individual meals	(13.5 - 85.1)	
Low-Protein - Low Calorie Diet		
Breakfast	18.0 ± 4.0 (5)*	
Lunch	6.3 ± 1.1 (6)	54.6
Dinner	30.3 ± 7.3 (3)	
Range for individual meals	(3.4 - 30.8)	

*Mean ± standard deviation; number of determinations in ( ).

Analysis for selenium: Selenium content in meals was determined by means of solvent extraction combined with activation analysis (15). The contents of each meal were homogenized in a blender and the total weight of the homogenate was recorded. A portion of the homogenized sample, 0.5 gm, was placed into a 100 ml round bottom flask to which was added 3 ml concentrated nitric acid. The flask was covered with either a watch glass or a Gooch type crucible and the samples were heated on a steam bath overnight. The samples were then evaporated to dryness on a rotary vacuum evaporator. Three ml of double distilled water was added to the residue, warmed on a steam bath (30 seconds) and then transferred to a 60 ml separatory funnel. The digestion flask was further rinsed with 2 ml water which was added to the separatory funnel.

For extraction, a drop of 0.1% brilliant yellow was used as an indicator and the solution was adjusted to pH 7.0 with 5 M ammonium hydroxide. Two mls of acetate buffer, 2 ml of 60% diethyldithiocarbamate and 5 ml CCl_4 were added to the mixture. The contents were vigorously shaken for 5 minutes. The CCl_4 layer was drawn off and placed into a test tube and put on a shaking hot water bath for approximately 30 minutes to evaporate the CCl_4. To the residue in the test tube was added one ml n-butylamine, mixed and then transferred into a 3 ml plastic vial. The vials were irradiated in a neutron activator and the contents

assayed for ^{77m}Se (16). Spectrographically pure selenium and National Bureau of Standard's Bovine Liver Powder #1577 were used as reference materials. The sensitivity of the method was 0.05 µg Se. The average chemical yield for 0.1, 0.3, and 0.6 µg Se was greater than 91% for the three concentrations observed. The chemical yield in the standards carried through the digestion was found to be of the order of 1-2% lower than those not subjected to acid digestion. Over a 3-year period, analysis of an internal control sample had a mean and standard deviation of 1.1 ± 0.06 µg Se per gm tissue. Using the National Bureau of Standards reference for selenium, the recovery was 1.1 ± 0.05 µg Se per gm tissue. The counting error was 2%. Results are expressed as µg Se per meal and µg Se per day. Samples were irradiated in the Omaha VA Hospital radiation center in a 18 KW TRIGA reactor, operating with a thermal neutron flux of 3.1 x 10" μ/cm^2 sec.

RESULTS

The mean selenium contents for regular meat-based meals were 29.2 ± 9.8 µg Se/meal (mean ± S.D.); 37.7 ± 6.7 for vegetarian, and 15.6 ± 3.2 for low protein-low calorie. The daily regular, vegetarian, and low protein-low calorie diets contained a total of 85.5, 118.1, and 54.6 µg of selenium, respectively (Table 1). The data in Table 2 show the percentages of the recommended daily intake provided by the 3 diets as related to the low and high extremes of the

TABLE 2

Percent of Recommended Daily Intake of Selenium Provided by the Different Diets

Diet	Per day µg	Low* 50 µg	High* 200 µg
		%RDA	
Regular (meat-based)	85.5	171	43
Vegetarian (lacto-ovo)	118.1	236	59
Low Protein-Low Calorie	54.6	109	27

*Low and high refer to the extremes of the estimated safe and adequate recommended range for selenium (7).

the recommended range. As shown, the percentages of the recommended daily intake provided by the 3 diets were above 100% when compared to the lowest level of 50 µg per day, while the percentages provided by the same diets ranged from 27 to 59% for the higher value of 200 µg per day. The actual intakes of selenium by the patients would be less than the values cited because frequently all the food was not consumed. Indeed, both the regular and vegetarian diets provided calories and protein in excess of the RDA while the low protein-low calorie diet did not (Table 3). Also reported in Table 3 are zinc contents, zinc-selenium ratios and the selenium concentration per 1000 Kcal for each diet. Zinc contents and zinc-selenium ratios were included because of suggestions that zinc and selenium interact in liver disease (17) and cancer (18). However, this relationship has not been established.

TABLE 3

Calories, Protein, Zinc, and Selenium/1000 Kcal

Diet	Calories Kcal/day	Protein gm/day	Zinc mg/day	Zn/Se ratios µg:µg/day	Se/1000 Kcal µg
Regular (meat-based)	3,156 ± 264	129 ± 15	14.6 ± 4.5	191 ± 24	27.1
Vegetarian (lacto-ovo)	3,103 ± 292	125 ± 12	12.2 ± 1.2	194 ± 80	38.1
Low protein-Low calorie	1,506 ± 181	45 ± 7	7.3 ± 1.8	155 ± 29	36.3

DISCUSSION

Since 1957, selenium has been known to be an essential trace element for several mammalian species. In animals, selenium deficiency leads to distinct symptoms such as: liver necrosis in rats (1), edema (exudative diathesis) in birds (2, 3), and muscle dystrophy (White Muscle Disease) in cattle, sheep, and horses (4, 19-21). In countries where the selenium content of soil and forage crops is low, White Muscle Disease is a serious problem. One report has suggested that selenium deficient subhuman primates suffer from a variety of conditions including alopecia, weight loss, degeneration of the liver, pancreas, and kidneys. Eventually they die in a profound marked state of constitutional disorder, general ill health, and malnutrition (22). This report needs confirmation since it was not established that the monkeys were not also vitamin E deficient (23).

Selenium has been shown to be an integral part of the enzyme glutathione peroxidase (24, 25); thus establishing a specific biochemical function for this element.

Recent reports from mainland China indicate that selenium supplementation to children aged 1-9 years showed remarkable preventive effect to Keshan's Disease, an endemic cardiomyopathy of unknown cause (26-28). It was suggested that the cause of this disease may be the lack of selenium which if not treated can be fatal. The fact that selenium helps prevent this disease adds evidence that selenium is essential for humans and thus necessary for maintaining normal health.

The total daily dietary intake of selenium has not been extensively studied in the United States. In 1967, Hopkins (29) analyzed diets from the Baltimore area by neutron activation analysis and found "nondetectable" selenium levels. Apparently, the methodology was not adequate to detect the rather low concentrations of selenium normally present. In 1970, Schroeder et al. (8) found, by analysis, 62 µg Se/day in hospital diets consumed over a 2 day period. The calculated intake of selenium ranged from 60-150 µg/day for adults living in the northeast area of the U.S. In 1974, the Market Basket Study by the Food and Drug Administration reported a calculated mean daily intake of 169 µg (30). The average daily intake of selenium consumed by a California adult male and female over a 30 day period was reported to be 107 and 99 µg, respectively. Selected food items were analyzed. However, the total daily diets were not analyzed but the type and quantity of each food consumed were recorded and the selenium level calculated (31). Calculated intakes for 8 other subjects ranged from 92 to 160 µg per day. In another study, the content of an omnivorous - university

cafeteria diet and a home-cooked lacto-ovo vegetarian diet, collected over a period of 7 days, averaged 93 µg Se/day for the omnivorous diet and 82 µg Se/day for the vegetarian diet. An "all-plant" experimental diet averaged 86 µg Se/day (32). In 1978, Zabel et al. (33) summarized daily dietary selenium intakes in the U.S. and various other countries. In general there is no evidence of reported selenium deficiency in humans in the U.S.

Recent data indicated that the average British diet provides approximately 60 µg of selenium per day (34). In Canada, Thompson et al. (35) reported an average daily intake of 168 µg selenium with a range of 113-220 µg. This was based on four composite diets from three cities. In New Zealand, where selenium concentrations are low in soil and selenium deficiencies in animals are well documented, the daily intake of selenium for humans is low and in one study averaged 56 µg (36). In a report from China, the average daily selenium intake of 12 women was only 10.5 µg (2.4 to 19.7 µg) (unpublished). In another study from New Zealand of four women who ate a normal diet for 14 days, daily intake of selenium averaged only 24.2 µg (37) substantially below U.S. National Research Council recommended daily allowance. Likewise, a report from Sweden indicated that daily intake of 67 year-old pensioners ranged from 8.7-96.3 µg and averaged 31 µg (38).

ACKNOWLEDGEMENTS

It is a pleasure to acknowledge Renato Ferretti for his dedicated assistance in preparation of the manuscript. We also thank Ellen D. Brown, Mareea Wilson, R. D. and the personnel of the VA Medical Center Dietetic Service, Washington, D.C. The helpful comments of Drs. Orville Levander and Kent Stewart are also appreciated.

REFERENCES

1. SCHWARZ, K. and FOLTZ, C. M. Selenium as an integral part of Factor 3 against dietary necrotic liver degeneration. J. Am. Chem. Soc. 79: 3292-3293, 1957.
2. SCHWARZ, K., BIERI, J.B., BRIGGS, G.M. and SCOTT, M.L. Prevention of exudative diathesis in chicks by Factor 3 and selenium. Proc. Soc. Exp. Biol. Med. 95:621-625, 1957.
3. PATTERSON, E.L., MILSTREY, R. and STOKSTAD, E.L.R. Effect of selenium in preventing exudative diathesis in chicks. Proc. Soc. Exptl. Biol. Med. 95: 617-620, 1957.
4. MUTH, O.H., OLDFIELD, J.E., REMMERT, L.F. and SCHUBERT, J.R. Effects of selenium and vitamin E on white muscle disease. Science 128: 1090, 1958.
5. McCOY, K. and WESWIG, P.H. Some selenium responses in the rat not related to vitamin E. J. Nutr. 98: 383-389, 1969.
6. THOMPSON, J.N. and SCOTT, M.L. Role of selenium in nutrition of the chick. J. Nutr. 97: 335-342, 1969.
7. FOOD AND NUTRITION BOARD. Recommended Dietary Allowances, Ninth Revised Edition, 1980. Washington, DC: Natl. Acad. Sci., 1980.
8. SCHROEDER, H.A., FROST, D.V. and BALASSA, J.J. Essential trace metals in man: Selenium. J. Chron. Dis. 73: 227-243, 1970.
9. GANAPATHY, S.N., JOYNER, B.T., SAWYERS, D.R. and HAFNER, K.M. Selenium content of selected foods. Third International Symposium in Trace Element Metabolism in Man and Animals. July, 1977, Freising - Weihenstapham, West Germany.
10. MORRIS, V.C. and LEVANDER, O.A. Selenium content of food. J. Nutr. 100: 1383-1388, 1970.
11. LEVANDER, O.A. Selenium and chromium in human nutrition. J. Am. Dietet. Assn. 66: 338-344, 1975.
12. SMITH, J.C., JR. and HALSTED, J.A. Clay ingestion (geophagia) as a

source of zinc for rats. J. Nutr. 100: 973-980, 1970.
13. CHURCH, C.F. and CHURCH, H.N. Food value of portions commonly used, 10th ed., Philadelphia, J. B. Lippincott Co., 1966 and USDA Agric. Handbook, No. 8, 1963, Watt, B. A. and Merrill, A. L., Composition of Foods - Raw, Processed, Prepared.
14. BROWN, E.D., McGUCKIN, M.A., WILSON, M. and SMITH, J.C., JR. Zinc in selected hospital diets. J. Am. Dietet. A. 70: 632-635, 1976.
15. HAHN, H.K.J., WILLIAMS, R.V., BURCH, R.E., SULLIVAN, J.F. and NOVAK, E.A. Determination of serum selenium by means of solvent extraction combined with activation analysis. J. Lab. Clin. Med. 80: 718-722, 1972.
16. BLOTCKY, A.J., ARSENAULT, L.J. and RACK, E.P. Optimum procedure for the determination of selenium in biological specimens using ^{77m}Se neutron activation. Anal. Chem. 45: 1056-1060, 1973.
17. SULLIVAN, J.F., BLOTCKY, A.J., JETTON, M.M., HAHN, H.K. and BURCH, R.E. Serum levels of selenium, calcium, copper, magnesium, manganese, and zinc in various human disease. J. Nutr. 109: 1432-1437, 1979.
18. SCHRAUZER, G.N., WHITE, D.A. and SCHWEIDER, C.J. Cancer mortality correlation studies IV: Associations with dietary intakes and blood levels of certain trace elements, notably Se-antagonist. Bioinorg. Chem. 7: 35-56, 1977.
19. SELENIUM AND HUMAN HEALTH. A Statement of the Food and Nutrition Board, Natural Research Council. Nutrition Reviews 34: 347-348, 1976.
20. NATIONAL RESEARCH COUNCIL, AGRICULTURAL BOARD, COMMITTEE ON ANIMAL NUTRITION, SUBCOMMITTEE ON SELENIUM: (J.E. Oldfield, Chairman) Selenium in Nutrition, Washington, D.C., National Academy of Sciences, 1971.
21. SELENIUM: Report of Committee on Medical and Biologic Effects of Environmental Pollutants National Research Council, National Academy of Sciences, Washington, D.C., 1976.
22. MUTH, O.H., WESWIG, P.D., WHANGER, P.D. and OLDFIELD, J.E. Effect of feeding a selenium-deficient ration to the subhuman primate (Saimiri sciureus). Am. J. Vet. Res. 34: 1603-1605, 1971.
23. BURK, R.F. Selenium in man. In: Trace Elements in Human Health and Disease, A.S. Prasad and D. Oberleas, eds., Academic Press, New York, 1976.
24. ROTRUCK, J.T., POPE, A.L., GANTHER, H.E., SWANSON, A.B., HAFEMEN, D.G. and HOEKSTRA, W.G. Selenium: Biochemical role as a component of glutathione peroxidase. Science 179: 588-590, 1973.
25. GANTHER, H.E., HAFEMAN, D.G., LAWRENCE, R.A., SERFASS, R.E. and HOEKSTRA, W.G. Selenium and Glutathione Peroxidase in Health and Disease - A Review. In: Trace Elements in Human Health and Disease, A.S. Prasad, ed., Academic Press, NY, 1976.
26. RESEARCH GROUP OF THE CHINESE ACADEMY OF MEDICAL SCIENCES: Observations on the effects of sodium selenite in prevention of Keshan Disease. Chinese Medical Journal 92: 471-475, 1979.
27. RESEARCH GROUP OF THE CHINESE ACADEMY OF MEDICAL SCIENCES: Epidemiological studies on the etiologic relationship of selenium and Keshan Disease. Chinese Medical Journal 92: 477-482, 1979.
28. EDITORIAL. Selenium in the heart of China. Lancet 2: 889-890, 1979.
29. HOPKINS, L.L., JR. and MAJAJ, A.S. Selenium in human nutrition. In: Selenium in Biomedicine, O.H. Muth, J.E. Oldfield and P.J. Weswig, eds., AVI Publishing Co., Westport, Conn., 1967, pp. 203-214.
30. U.S. BUREAU OF FOODS, FOOD AND DRUG ADMINISTRATION, COMPLIANCE PROGRAM EVALUATION. Federal Year 1974: Selected minerals in foods survey. Program Circular 7320, 08C, Aug. 25, 1975.
31. SCHRAUZER, G.N. and WHITE, D.A. Selenium in human nutrition; dietary intake and effects of supplementation. Bioinorg. Chem. 8: 303-318, 1978.
32. GANAPATHY, S. and DHANDA, R. Selenium content of omnivorous and vegetarian diets. Fed. Proc. 35: 360, 1976.
33. ZABEL, N.L., HARLAND, J., GORMICAN, A.T. and GANTHER, H.E. Selenium content of commercial formula diets. Am. J. Clin. Nutr. 31: 850-858, 1978.

34. THORN, J., ROBERTSON, J., BUSS, D.H. and BUNTON, N.G. Trace nutrients, selenium in British food. Br. J. Nutr. 39: 391-396, 1978.
35. THOMPSON, J.N., ERDODY, P. and SMITH, D.C. Selenium content of food consumed by Canadians. J. Nutr. 105: 274-277, 1975.
36. WATKINSON, J.H. The selenium status of New Zealanders. New Z. Med. J. 80: 202-205, 1974.
37. STEWART, R.D., GRIFFITHS, N.M., THOMSON, C.D. and ROBINSON, M.F. Quantitative selenium metabolism in normal New Zealand women. Brit. J. Nutr. 40: 45-54, 1978.
38. ABDULLA, M., KOLAR, K. and SVENSSON, S. Selenium. In: Nutrition and Old Age. Scand. J. Gastroenterology 14: 181-184, 1979. Supplement 52.

NUTRITION RESEARCH, Vol. 1, pp. 243-252, 1981
0271-5317/81/030243-10$02.00/0 Printed in the USA.

AN EVALUATION OF DRIED SKIMMED MILK ON CHILDREN'S GROWTH IN KHARTOUM PROVINCE, SUDAN

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ABSTRACT

In a controlled trial mothers and children attending urban maternal and child health (MCH) clinics in Khartoum Province were given a fortnightly take home food supplement of 1 Kilogram of dried skimmed milk (DSM) or of local beans. There were approximately 300 children aged 6-26 months in each group and each child was followed for 3 to 6 months. A comparison of the two groups showed the most important findings were

a) The average monthly weight gain was found to be very similar in the DSM and Beans groups, but for height gain the DSM group did significantly better.

b) Those children most under-weight for their age at the start showed the greatest gains during the trial, whereas height gains were not related to initial heights.

c) The children's age and sex did not appear to have a significant effect on weight or height gains.

d) About 40% of children showed an improvement in weight-for-age and weight-for-height categories and only 5% a deterioration. There was no difference between the DSM and Beans groups using these categories.

e) When breast-feeding was continued throughout the trial, the DSM group children showed significantly greater weight gains, whereas in those not breastfed at all the Beans group did significantly better. The better height gain in the DSM group appeared to be unrelated to breast-feeding practices.

KEY WORDS: Supplementary feeding, Food aid, Growth, Breastfeeding, Controlled trial.

INTRODUCTION

The benefits of 'take home' supplementary child feeding programmes have been questioned in a recent review. There has also been criticism of the distribution of dried skimmed milk (DSM) in the Third World because of its possible adverse effects on breast-feeding practices and child growth, and in causing illness and infant deaths (1).

This issue is a separate one from that concerning the promotion of infant milk formulae by industry, but the information given here is obviously relevant and closely related to that problem (2,3).

DSM is widely used in food aid programmes (4) and supplementary feeding programmes (5). In another publication (6) we have attempted to evaluate some of the adverse effects which have been claimed for DSM but in this publication we examine the relationship of DSM and breast-feeding on child growth.

The results are from a controlled trial carried out in 1978/79 in Khartoum Province, Sudan. The evaluation focusses on the relationship between breast-feeding and gains in weight and height of the children of one group of mothers who received a take home supplement of DSM compared to another group given instead an equivalent weight of local beans.

METHODS

These have been more fully outlined in previous publications (6,7). The trial was carried out in 8 different mother and child health (MCH) clinics at health centres in Khartoum, Khartoum North and Omdurman, of which 4 pairs were formed by geographical area and one of each pair was then allocated for the distribution of DSM and one for Beans. Mothers attending the MCH clinics for the first time with a child aged 6-26 months were accepted into the trial. Of a total of 646 children accepted 626 (97%) were eventually followed up for between 3 and 6 months. Of the 626 children who continued throughout the trial 287 were in the DSM group and 339 in the Beans group. Weight and height were measured at the clinics by a trained female nutrition worker using UNICEF beam scales and infantometers at the beginning and end of the child's period of follow-up.

Within a few days of accepting the mother into the trial a home visit was made by the nutrition worker to collect information on breastfeeding practices, socio-economic and demographic information, and on the household conditions.

The mother and child were followed at 2 week intervals (alternating by clinic and home visits) and the mother was given 1 kg of DSM or Beans on each occasion. At each follow-up the mother was asked if the child had had diarrhoea, fever or vomiting over the previous 2 week interval, and if so what health institutions the child had been taken to.

At the beginning and end of the trial the mother was asked to assess her child's appetite and activity as good, normal or poor.

RESULTS

There was no significant difference in the children's age distribution in the two groups and there was an approximately equal proportion of both sexes, with the males 49% of the DSM group and 53% of the Beans group. The fortnightly follow-ups were more regular and more frequent in the Beans group with 81.4% being seen at the correct interval compared with 71.4% of the DSM mothers.

Of all the mothers accepted into the trial less than 5% missed more than two of the monthly clinic follow-ups, and only 2.4% did not answer both the first and final questionnaire on breast-feeding. Only 6% of children did not have a final complete set of height and weight measurements.

A comparision of the socio-economic and demographic data showed that the DSM group children appeared to be at a significant disadvantage in terms of the lower educational background of the parents, a lower family expenditure on food and poorer household conditions (7). Fewer Beans group mothers (64.9%) were breast-feeding at the start of the trial compared to DSM (78.4%) ($p<0.01$). Approximately 70% of both groups were giving supplements of fresh cow's and/or goat's milk to their children at the onset of the trial.

Weight gain in children

The weights of the children on entry into the study were much as would be expected for a semi-urbanised group of children from a city such as Khartoum with the females being on average about 0.5kg lighter. However, the children in the DSM group were significantly lighter than those in the Beans group, although the overall difference was only 0.23kg. (Table 1, Analysis of variance $p<0.05$, taking age groups and sex into account.)

TABLE 1

Children's average initial weights (Kgs) and heights (Cms) on entry to the trial, by group, age and sex.

Initial Age (months)	Average Weight (Kgs)				Average Height (Cms)			
	DSM		Beans		DSM		Beans	
	M	F	M	F	M	F	M	F
	n=143	n=148	n=181	n=156	n=142	n=148	n=165	n=137
6 - 8	7.81	7.08	7.77	7.23	68.9	67.0	66.9	66.4
9 - 11	8.23	8.24	8.98	8.00	72.5	71.4	73.8	71.4
12 - 14	8.90	8.54	9.23	8.67	74.2	73.5	74.2	75.1
15 - 17	9.21	8.90	9.89	9.82	77.1	76.0	77.6	79.2
18 - 20	9.89	9.47	10.61	9.13	79.4	77.7	78.9	77.7
21 - 23	10.89	10.46	9.66	10.48	80.9	82.1	78.7	82.8
24 - 26	9.64	9.40	11.29	10.85	81.2	80.5	86.9	82.7
Total	8.91	8.48	9.18	8.62	74.7	73.3	74.0	74.0

In order to control for the effects of age and the different months of individual child follow-up the average weight gain per month for each child was found by subtracting the initial weight from the final weight and dividing by the final number of months of follow-up.

Table 2 shows the average weight gains per month in the DSM and Beans groups by age groups and for the sexes combined. An analysis of covariance was carried out taking into account the initial weight at entry into the trial (which was not the same in the two groups), age and sex. This showed that the weight gain per month:-

a) was inversely related to the initial weight ($p<0.001$), which meant that lighter children for their age tended to have a larger monthly gain in weight (and vice versa).

b) was not significantly different for male or female children ($p>0.05$).

c) was not different between the DSM and Beans groups ($p>0.4$).

d) was similar for both the DSM and Beans Groups ($p>0.3$).

Table 2 also shows the average initial weight for each age group as a percentage of the 50th Centile of the NCHS standards (8) and the expected monthly weight gains are a 5 month interval based on these standards. This shows that in both DSM and Beams groups the monthly gains were greater than those expected in a standard population of children. The 'catch-up' effect was greatest in those children who were oldest when they entered the trial.

TABLE 2

Average initial weight (Kg) as a percentage of expected, by age groups, together with the expected and observed mean monthly weight gains in the DSM and Beans groups (sexes combined).

Initial Age (months)	Mean weight at entry to trial	Mean weight gain per month (Kg)		
	% of expected	expected	observed	
			DSM	Beans
6 - 8	92	0.38	0.43	0.36
9 - 11	90	0.26	0.39	0.39
12 - 14	88	0.19	0.38	0.29
15 - 17	87	0.19	0.35	0.33
18 - 20	88	0.19	0.34	0.35
21 - 23	85	0.18	0.30	0.32
24 - 26	84	0.17	0.23	0.33

(Expected weights, expected heights and expected gains have been calculated from NCHS (DHEW 1970) standards using the 50th centile. Expected gains calculated over 5 month interval starting from midpoint of each initial age group.)

Height gain of children

The same analytical approach was used for average height gain as for weight. The average initial heights for males and females were within the expected ranges for the age groups (see Table 1) and the heights showed no significant difference between the DSM and Beans groups with the sexes combined ($p>0.05$).

Table 3 shows the average height gains per month in the DSM and Beans groups by age compared to the expected. An anlysis of covariance showed that the average height gain per month:-

a) was not related to the initial height at entry to the trial ($p>0.1$)

b) was similar for males and females ($p>0.5$)

c) varied between the age groups and although this was just significant ($p<0.05$), there was no particular pattern

d) the DSM group children grew on average 0.24cm per month more than those in the Beans group, this was a highly signficant difference ($p<0.001$).

TABLE 3

Average height (cm) as a percentage of expected, by age groups, together with the expected and observed mean monthly height gains in the DSM and Beans groups (sexes combined).

Initial Age (months)	Mean height at entry to trial	Mean height gain per month (Cm)		
	% of expected	expected	observed DSM	observed Beans
6 - 8	100	1.38	0.88	0.46
9 - 11	101	1.20	0.77	0.57
12 - 14	97	1.16	0.75	0.48
15 - 17	98	0.88	0.56	0.46
18 - 20	96	0.90	0.74	0.57
21 - 23	94	0.87	0.51	0.49
24 - 26	97	0.80	0.97	0.69

An analysis of initial heights compared to a standard child population (Table 3) showed a greater average deficit in the older age groups. The average height gains were substantially less than expected in the reference population and an increase in stunting with increasing age is therefore to be expected (9).

Weight for age and weight for height categories

Only children who had had five months or more of completed follow-up were included in this analysis in order to overcome the problem of variable lengths of follow-up and to allow time for detectable changes to have occurred. An individual child's weight for age (weight/age) and weight for height (weight/height) were examined at the start and end of the follow-up period and expressed as a percentage of the median standard using the NCHS standards (8). Weight/age and weight/height were divided into 4 broad categories: less than 60% of reference; 60-79%; 80-99%; 100% or more. Children were classified as better or worse if they moved up or down by one or more categories over the 5 months of the trial.

Table 4 shows that the DSM group children were slightly lighter than the Beans group in weight/age at the start of the trial and that the DSM group had a higher percentage with weight/ height of less than 80% standard. However, at the end of the 5 months of follow-up the weight/ age and weight/height distributions between the two groups were very similar (Table 5).

For weight/age and weight/height indices the cut-off point dividing 'normal' from 'malnourished' is usually taken as 80% of the reference median, approximately equivalent to the 10th centile. Table 5 shows that at the end of the trial approximately 10% of children were below this cut-off point in weight/age whereas the proportion below 80% weight/ height is not significantly different from the number to be expected in the general population. It is evident that most of the deficit in weight for age is therefore due to stunting in height.

TABLE 4

Percentage of children (with 5 months or more of follow-up in the DSM and Beans groups by initial weight-for-age and weight-for-height categories.

Category	Weight for age		Weight for height	
	DSM n=268	Beans n=157	DSM n=267	Beans n=126
	%	%	%	%
60% or less	1.1	1.9	0.7	0.8
60-79%	24.6	20.4	13.5	9.5
80-99%	58.2	54.8	53.9	61.9
100% or more	16.0	22.9	31.8	27.8
	99.9	100.0	99.9	100.0

TABLE 5

Final weight for age and weight for height categories for children who had 5 months follow-up or more.

Final category at end of follow-up	Weight for age		Weight for height	
	DSM n=268	Beans n=157	DSM n=267	Beans n=126
	%	%	%	%
60% or less	0	0	0	0
60-79%	11.6	9.6	4.1	1.6
80-99%	48.9	45.2	30.7	33.3
100% or more	39.5	45.2	65.2	65.1
	100.0	100.0	100.0	100.0

The individual children's categories at the start and at the end of the 5 month follow-up period were compared to see whether they were worse, the same or improved (Table 6). This also showed no difference between the DSM and Beans groups. About 55% of children in both groups showed no change, approximately 40% showed an improvement of one or more categories and only about 5% showed a deterioration.

It was concluded that there was no evidence for any difference in the response of the two groups as judged by changes in weight for age and weight for height categories.

TABLE 6

Average monthly weight and height gains in the DSM and Beans groups according to breast-feeding practices during the trial (sexes combined and number of subjects shown in brackets).

Possible change of category at end of follow-up	Weight for age		Weight for height	
	DSM n=268	Beans n=157	DSM n=267	Beans n=126
	%	%	%	%
Worse	6.0	8.3	3.8	3.2
No change	53.7	52.2	52.4	53.2
Improved	40.3	39.5	43.8	43.6
	100.0	100.0	100.0	100.0

Breast-feeding practices and average weight and height gain

The effect of breast-feeding practices on weight and height gains was analysed by dividing the children into 3 categories according to whether they were not breast-fed at all (Not BF) during the trial, were breast-fed at the start but stopped during the trial (BF stopped) or were breast-fed throughout (BF throughout).

It can be seen from Table 7 that the differences in weight gain per month between the DSM and Beans group was different in the 3 categories. Analyses of covariance to compare the weight gains in each category were performed separately, taking into account initial weight, age and sex. These showed that amongst the children not breast-fed at all (Not BF) the weight gain was significantly higher for the Beans group ($p<0.01$), while the reverse was true for those breast-fed throughout (BF throughout) when the DSM group did significantly better ($p<0.001$). There was no difference between DSM and Beans for those that stopped breast-feeding during the trial (BF stopped).

TABLE 7

Average monthly weight and height gains in the DSM and Beans groups according to breast-feeding practices during the trial (sexes combined and number of subjects shown in brackets).

Breast-feeding practice (BF)	Average weight gain per month (Kgs)		Average height gain per month (Cms)	
	DSM	Beans	DSM	Beans
Not BF	0.26 (61)	0.38 (117)	0.77 (61)	0.55 (105)
BF stopped	0.32 (47)	0.30 (51)	0.66 (46)	0.44 (37)
BF throughout	0.42 (180)	0.33 (169)	0.78 (180)	0.50 (160)
Total	0.37 (288)	0.34 (337)	0.76 (287)	0.51 (302)

It can also be seen from Table 7 that in each of the 3 breast-feeding categories the height gain was significantly ($p<0.001$) better in the DSM group, with an average difference of 0.25cm per month. It thus appears that the difference in weight gain between the DSM and Beans groups depended on whether or not the child was being breast-fed. The difference in height gain, however, was apparently independent of breast-feeding practice.

DISCUSSION

In this study the DSM and Beans were given to the mother fortnightly to 'take home' and we have no means of knowing what actually happened to these food supplements. Also it needs emphasising that this trial compared DSM with another food, locally purchased beans, and not with an untreated or 'pure' control group. It was felt to be unethical to withdraw a food supplement from mothers coming to clinics where DSM had been distributed for several years under the auspices of a voluntary organisation. It was also quite clear that attendance at the clinics was strongly encouraged by such supplements. The DSM and Beans groups were not strictly comparable on several important background variables and so the results must be viewed with some caution. Also much of the information had to be collected by interviewers using questionnaires based on memory recall. In the measurement of weight and height, the analysis revealed considerable variation, despite training the nutrition workers and close supervision, which has the effect of reducing the likelihood of finding any differences.

The findings on weight and height gains are difficult to interpret. The few studies which have shown an effect of 'take home' supplementary feeding have found an increase in weight but not in height (1). If we had not taken the individual children's initial weights into account in the analysis, we might also have concluded that DSM did better than Beans. Poorly selected controls could easily lead to there being considerable differences in initial weights between two groups and it is recognised that those children most underweight for their age are most likely to make the greatest short-term gains. However, it must be emphasised that approximately 40% of children in both DSM and Beans groups showed an improvement over the five months follow-up period in weight/age and weight/height, whereas only about 5% showed a deterioration. Since there

was not a 'pure' control group it is not possible to say what would have happened to children of mothers not receiving a supplement of either DSM or Beans.

Baby milk substitutes and DSM are widely available in many developing countries, particularly in the urban areas. There are many claims linking bottle feeding with diarrhoea, malnutrition and infant deaths (2,3,10) but soundly based community studies are very few and the number of well controlled trials are even fewer (1). Supplementary programmes for children are usually justified on their supposed nutritional benefits or impact on child growth. However, it is precisely these benefits that have been the most poorly evaluated whilst the possible harmful effects have received wide publicity and attention (5). This situation can only be improved in the long term if more attention is given to implementing good scientific studies to evaluate and quantify these supposed benefits and harmful effects.

ACKNOWLEDGEMENTS

This study only became possible because of a generous financial grant from the Overseas Development Administration (ODA) of the United Kingdom government. We owe many thanks to the Sudanese Ministry of Health and the Assistant Commissioner of Health for Khartoum Province who were very helpful in loaning support, staff and equipment.

Our thanks also go to Miss Samira Hamo of the Ministry of Health and Miss Sana Arbab, of the Ministry of Social Affairs, who supervised the field work; to Shirley Harrison for all her special efforts in computing and analysing this study and to Michael Elston for advice on the analysis of the weight and height data.

REFERENCES

1. BEATON G.H. and GHASSEMI H. Supplementary Feeding Programmes for Young Children in Developing Countries. Report prepared for UNICEF and the ACC Sub-Committee on Nutrition of the United Nations. UNICEF, New York, USA. 1979.

2. GREINER T. Regulation and Education: Strategies for Solving the Bottle Feeding Problem. Cornell International Nutrition Monograph Series, Number 4. Division of Nutritional Sciences, Cornell University, Ithaca, N.Y., U.S.A. 1977.

3. WORLD HEALTH ORGANIZATION. Statement and recommendation on infant and young child feeding. Development Dialogue, 1980:1, 102-119. 1980.

4. MAXWELL S.J. and SINGER H.W. Food Aid to Developing Countries: A Survey. Discussion paper published by the Institute of Development Studies, University of Sussex, Brighton, U.K. 1978.

5. MAXWELL S. Food aid for supplementary feeding programmes. Food Policy, November 1978, 289-298. 1978.

6. ZUMRAWI F., VAUGHAN J.P., WATERLOW J.C., KIRKWOOD B.R. Dried skimmed milk, breast-feeding and illness episodes - a controlled trial in young children in Khartoum Province, Sudan. International Journal of Epidemiology - accepted for publication. 1981.

7. VAUGHAN J.P., WATERLOW J.C., ZUMRAWI F., HASSAN M.M., HAMO S., ARBAB S., KIRKWOOD B., HARRISON S., Report on the Pilot Trial to Determine Some of the Effects of Distributing Dried Skimmed Milk in Mother and Child Health Clinics in Khartoum and Omdurman, Sudan. Published by the Ross Institute and the Department of Human Nutrition, London School of Hygiene and Tropical Medicine, U.K. 1980.

8. DEPARTMENT OF HEALTH, EDUCATION AND WELFARE (DHEW). The NCHS Growth Curves for Children Birth to 18 Years (United States) by Hamill P.V.V., Drizd T.A., Johnson C.C., Reed R.B., Roche A.F., DHEW Publication PHS-78-1650, Hyatsville, Maryland, U.S.A. 1977.

9. WATERLOW J.C., BUZINA R., KELLER W., LANE J.M. , NICHAMAN M.Z., TANNER J.M. The presentation and use of height and weight data for comparing the nutrition status of groups of children under the age of 10 years. Bulletin of the World Health Organization, 55, 489-498. 1977.

10. CHETLEY A. The Baby Killer Scandal. Published by War on Want, London, U.K. 1979.

NUTRITION RESEARCH, Vol. 1, pp. 253-259, 1981
0271-5317/81/030253-07$02.00/0 Printed in the USA.
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ADENINE-INDUCED OROTIC ACIDURIA IN RATS FED DIETS WITH AND WITHOUT OROTIC ACID

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ABSTRACT

Diets with 0, 0.05, 0.25, and 1% adenine sulfate, each with and without 1% orotic acid, were fed for 10 days to female weanling rats, 5 animals per treatment. In the absence of dietary orotic acid, urinary excretion of orotic acid was increased 8-fold (0.09 to 0.73 mg/day) by 0.25% adenine sulfate. In the presence of dietary orotic acid, excretion was 50 times greater and was enhanced 3-fold (4.4 to 12.7 mg/day) by 0.25% adenine sulfate. Adenine sulfate at 0.05% was ineffective at stimulating orotic acid excretion; adenine sulfate at 1.0% was as effective as 0.25% but led to symptoms of adenine toxicity. The two high levels of adenine also prevented development of fatty livers induced by orotic acid. Thus, the prevention of such fatty livers is associated with increased excretion of orotic acid. The orotic aciduria could be due to competition for renal reabsorption by adenine metabolites but more likely results from competition between pyrimidine and purine metabolism in the liver.
Key words: orotic acid; adenine; fatty liver; orotic aciduria

INTRODUCTION

In 1955, Standerfer and Handler (1) first reported that dietary orotic acid induces severely fatty livers in rats and shortly thereafter others (2) showed that adenine prevented and reversed such fatty livers. In the years since, the metabolic basis for these observations has formed the focus of numerous reports. Lipid accumulation occurs because lipoprotein maturation and secretion are inhibited (3-5). Upon orotic acid ingestion, hepatic pyrimidine nucleotides are elevated and purine nucleotides are concurrently depressed (6, 7); pyrimidines are increased because orotic acid is their precursor and purines are decreased presumably due to competition for a common substrate, phosphoribosylpyrophosphate. Adenine largely restores the balance between purine and pyrimidine nucleotides (6, 8), and may thus prevent and reverse the fatty liver induced by orotic acid. However, the metabolite responsible for the inhibition of lipid secretion and the target enzyme or process remain to be delineated.

A high degree of specificity is shown with respect to orotic acid as the cause of the fatty liver, adenine as the preventative agent, and the rat as the susceptible species. Orotic acid may be replaced by dihydroorotic acid, its immediate precursor (7), but not by other pyrimidines such

as uridine (7) or uracil and cytosine (2). An adenine precursor, 4-amino-5-imidazole carboxamide, protects (9) but other purines (guanine, hypoxanthine, and xanthine) do not (2). The rat appears to be unique in its susceptibility to orotic acid induced fatty liver as the following species do not so respond: mouse (10-13), hamster (10, 12), guinea pig (10, 12), chicken (11, 12, 14, 15), rabbit (12), pig (10) and monkey (10, 11). This high degree of species specificity suggests that human consumers of orotic acid, which is present in cow's milk (16), would not develop hepatic steatosis.

In pursuing the metabolic differences that determine the species specificity, Durschlag and Robinson (10) discovered that urinary excretion of dietary orotic acid varies markedly. The mouse excretes much more than the rat and the increased excretion may account for its lack of susceptibility to orotic acid induced fatty liver. This observation suggested the possibility that in the rat the protection afforded by adenine was accompanied by enhanced urinary excretion. Alternately, adenine could be promoting orotic acid catabolism, as by the usual degradation of free pyrimidines. The purpose of this work was to distinguish between these alternatives. Its pertinence is underlined by the fact that previous research (17) on this point was ambiguous; increased excretion was noted in one experiment, decreased in another, and no satisfactory explanation of the inconsistency was made. Accordingly, the present research was designed to examine the effect of adenine on urinary excretion of orotic acid in rats consuming diets with and without orotic acid. The dose response and the time course of the effects were concurrently studied as these had not been previously reported.

MATERIALS AND METHODS

The experiment was of a 4 x 2 factorial design (4 levels of adenine and 2 of orotic acid) with 5 female weanling albino rats (Harlan Sprague-Dawley) on each of the dietary treatments. Adenine at 0.00, 0.05, 0.25, and 1.00% adenine sulfate dihydrate (ICN Pharmaceuticals) represented null, suboptimal, optimal, and supraoptimal values with respect to preventing the fatty liver. Anhydrous orotic acid (Sigma Chemical Company) was fed at 0 and 1%, the latter known to induce fatty livers in rats within a week (7). Adenine and/or orotic acid were added to the basal diet previously described (7).

The animals were housed individually in stainless steel metabolism cages in a room with controlled temperature (20-22°) and lighting (10 hours light: 14 hours dark). The animals had access to water continuously but to food only between 1600 and 1000 hours. Twenty-four hour urine was measured at 1000 hours daily, diluted with distilled water washings of urine/feces separator and frozen for subsequent analysis (18). The animals were adapted to the feeding regimen and environmental conditions for 4 days on the basal diet; five were then randomly assigned to each treatment and monitored for 10 days. On day 10, the animals were sacrificed by decapitation, their livers removed, weighed, and frozen for lipid analysis (19).

All data were subjected to analysis of variance and the method of least-significant-difference was used to assess differences between treatment means at the 5% confidence level (20). For orotic acid excretion, the groups with and without orotic acid were treated separately because of a large difference in their standard errors.

RESULTS AND DISCUSSION

The results from the experiment are summarized in Table I. Feed efficiency as measured by the ratio of food consumed to weight gained was compared between treatments. Analysis of variance revealed no effect of orotic acid but an effect of adenine attributable to the 1% adenine sulfate diets only. The poorer feed efficiency noted was due to decreased intake and, to a greater extent, lower gain. This is attributable to the toxicity of adenine, which has an LD_{50} of 745 mg/kg (21). Since the rats consuming 1% adenine sulfate were consuming 500 mg adenine/kg daily, toxicity symptoms were expected. At the termination of the experiment, kidneys from these animals were enlarged and yellow, typical of such toxicity and the attendant excretion of the adenine derivative, 2, 8-dioxyadenine (22). Daily urine

TABLE I.

Several Effects in Rats Ingesting Adenine and Orotic Acid for 10 Days[a].

Treatment[b]						
Adenine sulfate	Orotic acid	Feed efficiency[c]	Urine volume[d]	Liver lipid[e]	Liver weight[f]	Urinary orotic acid[g]
0.00	0	2.16^{0}	4.5^{0}	5.2^{0}	4.58^{0}	0.09^{0}
0.05	0	1.95^{0}	4.4^{0}	5.1^{0}	4.49^{0}	0.18^{0}
0.25	0	2.03^{0}	8.4^{0}	5.5^{0}	4.44^{0}	0.79^{1}
1.00	0	2.85^{1}	13.6^{1}	5.9^{0}	4.53^{0}	0.73^{1}
0.00	1	2.26^{0}	3.6^{0}	17.6^{2}	5.74^{1}	4.44^{2}
0.05	1	2.15^{0}	5.0^{0}	11.7^{1}	5.49^{1}	6.89^{2}
0.25	1	1.96^{0}	5.7^{0}	5.6^{0}	4.85^{0}	12.74^{3}
1.00	1	3.42^{1}	14.4^{1}	5.3^{0}	4.39^{0}	13.81^{3}

[a] Means in a column not sharing a common superscript number are significantly different at $P < 0.05$.

[b] Treatments are denoted by percentage of diet as adenine sulfate and/or orotic acid with 5 animals per treatment.

[c] For 10 days, g diet consumed/g weight gained; SEM range: 0.06-0.26.

[d] For 10 days, ml urine/day; SEM range: 1.4-1.6.

[e] On day 10, liver lipid as percentage of liver wet weight; SEM range: 0.2-3.3.

[f] On day 10, liver weight as percentage body weight; SEM range: 0.07-0.22.

[g] Mean for days 2, 6, and 10 in mg/day; SEM range: 0.02-0.17 for 0% orotic acid and 0.56-1.26 for 1% orotic acid treatments. For the latter, excretions as a percentage of ingested dose correspond to 4.7, 7.0, 13.4, and 19.7, respectively.

volume (Table I) reflected the pattern of feed efficiency with the 1% adenine rats excreting much more urine than the others, another response to adenine's toxicity. Our values correspond with those of Clifford and Story (22). This parameter, as the feed efficiency, was unaffected by orotic acid.

With respect to liver lipid, adenine had no effect when orotic acid was absent from the diet. However, when orotic acid was present, increasing adenine prevented fatty liver development (Table I). Adenine sulfate at 0.05% reduced lipid accumulation somewhat, and 0.25% or 1.00% adenine sulfate lowered it to the control values. The effectiveness of 0.25% adenine sulfate was anticipated from previous studies (2-5, 8, 17), but no dose response had been previously reported. The partial effectiveness of the 0.05% level will be discussed later. Hepatomegaly often accompanies the hepatic steatosis induced by orotic acid (7). Such was the case in this experiment as liver weight was increased when fat accumulated, on 1% orotic acid with 0.00 or 0.05% adenine sulfate (Table I).

Orotic acid excretion was increased by adenine whether or not orotic acid was included in the diet (Table I). When orotic acid was absent, adenine sulfate at 0.25 and 1% led to an 8-fold increase over the 10 day experiment, while 0.05% did not significantly increase orotic acid excretion. Milner (23) has recently shown that 0.3% adenine sulfate induced orotic aciduria. When orotic acid was present in the diet, 16 to 50 times more orotic acid was excreted at the corresponding adenine levels; however, the high adenine levels retain their effectiveness with a 3-fold increase in urinary excretion. Thus, adenine levels effective in preventing the orotic acid fatty liver led to greater urinary excretion of orotic acid. Adenine does not exert its effect by stimulating orotic acid catabolism.

Figure 1 reveals the time course of orotic acid excretion in the absence of dietary orotic acid. With no adenine, excretion is low and constant throughout the study; on 0.05% adenine sulfate, a transient increase is noted in the first three days before it returns to normal; on 0.25 and 1% adenine sulfate the excretion is elevated and constant from day 2 on.

In figure 2, excretion upon consumption of dietary orotic acid is followed over 10 days. Without adenine, orotic acid excretion is constant around 4.4 mg/day; this is 50 times more than in absence of dietary orotic acid and corresponds to 5% of ingested dose. On 0.05% adenine sulfate, the values tend to be higher on day 6 and 10 but were not significantly so; if this tendency were real, it could explain the slight reduction (or delay) in fat accumulation on this diet. On 0.25 and 1% adenine, orotic acid excretion reached nearly 18 mg/day on day 10; this corresponds to around 20% of the dose and approaches the 27% excreted by the mouse (10), a species not susceptible to orotic acid induced fatty livers.

This paper presents evidence for an association in the rat between the prevention of orotic acid fatty liver and enhanced urinary excretion of orotic acid. It is evident that if more is excreted, less remains in the organism to alter hepatic metabolism such that fat accumulates. It is not clear however that adenine prevents the fatty liver primarily by stimulating renal clearance of orotic acid. While it is possible that adenine or one of its metabolites alters kidney function in such a way, it is more likely that the primary site of action is the liver. Adenine partially restores the balance of purine and

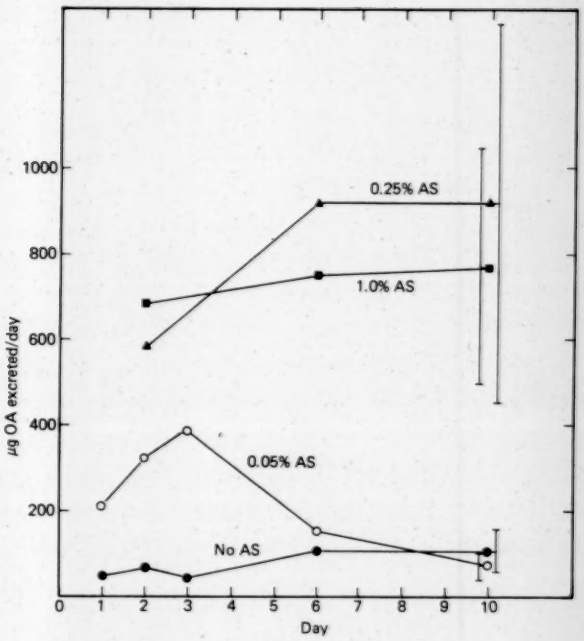

FIG. 1

Time course of orotic acid (OA) excretion in the absence of dietary orotic acid. Four levels of dietary adenine sulfate (AS) are denoted; means are for 5 rats; SEM are indicated for day 10 only.

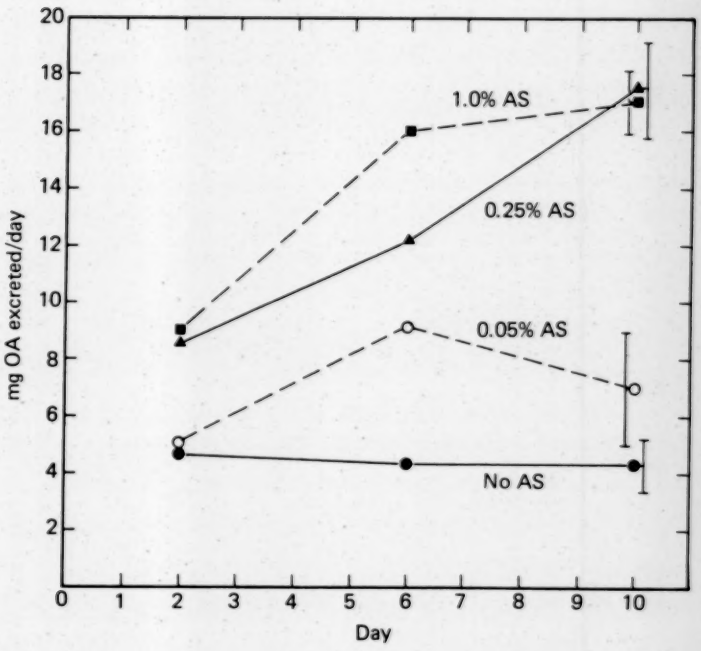

FIG. 2

Time course of orotic acid (OA) excretion in the presence of 1% dietary orotic acid. Four levels of diet adenine sulfate (AS), denoted; means are for 5 rats; SEM are indicated for day 10 only.

pyrimidine nucleotides (6, 8), less orotic acid is metabolized by liver and more is excreted in urine. Increased urinary orotic acid signals decreased hepatic utilization and, in this situation, any detrimental ramifications of its metabolism.

ACKNOWLEDGEMENTS

The author acknowledges research support from the Illinois Agricultural Experiment Station and the National Dairy Council. He thanks Helen Hegarty, Zania Shular, and Charles Matza for technical assistance, Mark Rorick for statistical computations, and Delores Menefee for typing the manuscript.

REFERENCES

1. STANDERFER, S. B. and HANDLER, P. Fatty liver induced by orotic acid feeding. Proc. Soc. Exp. Biol. Med., 90: 270-271, 1955.

2. HANDSCHUMACHER, R. E., CREASEY, W. A., JAFFE, J. J., PASTERNAK, C. A. and HANKIN, L. Biochemical and nutritional studies on the induction of fatty livers by dietary orotic acid. Proc. Nat. Acad. Sci. USA, 46: 178-186, 1960.

3. WINDMUELLER, H. G. Depression of plasma lipids in the rat by orotic acid and its reversal by adenine. Biochem. Biophys. Res. Comm., 11: 496-500, 1963.

4. WINDMUELLER, H. G. and LEVY, R. I. Total inhibition of hepatic β-lipoprotein production in the rat by orotic acid. J. Biol. Chem., 242: 2246-2254, 1967.

5. SABESIN, S. M., FRASE, S. and RAGLAND, J. B. Accumulation of nascent lipoprotein in rat hepatic golgi during induction of fatty liver by orotic acid. Lab. Invest., 37: 127-135, 1977.

6. VON EULER, L. H., RUBIN, R. J. and HANDSCHUMACHER, R. E. Fatty livers induced by orotic acid. II. Changes in nucleotide metabolism. J. Biol. Chem., 238: 2464-2469, 1963.

7. DURSCHLAG, R. P. and ROBINSON, J. L. Orotic acid-induced metabolic changes in the rat. J. Nutr., 110: 816-821, 1980.

8. MARCHETTI, M., PUDDU, P. and CALDARERA, C. M. Metabolic aspects of orotic acid fatty liver. Nucleotide control mechanisms of lipid metabolism. Biochem. J., 92: 46-51, 1964.

9. HAYANO, K., TSUBONE, T., AZUMA, I. and YAMAMURA, Y. The prevention of orotic acid fatty liver by the administration of 5-aminoimidazole carboxamide. J. Biochem. (Tokyo), 52: 379-380, 1962.

10. DURSCHLAG, R. P. and ROBINSON, J. L. Species specificity in the metabolic consequences of orotic acid consumption. J. Nutr., 110: 822-828, 1980.

11. VALLI, E. A., SARMA, D. S. R. and SARMA, P. S. Species specificity in orotic acid induced fatty liver. Ind. J. Biochem., 5: 120-122, 1968.

12. WINDMUELLER, H. G., LEVY, R. I. and SPAETH, A. E. Selective inhibition of hepatic but not intestinal β-lipoprotein production and triglyceride transport in rats given orotic acid. Adv. Exp. Med. Biol., 4: 365-375, 1969.

13. FURUNO, K., SHIMAKAWA, K. and SUZUOKI, Z. Effects of nutritional factors on the development of ethanol-induced fatty liver in KK and KK-A^y mice. J. Nutr., 105: 1263-1268, 1975.

14. BLOOMFIELD, R. A., LETTER, A. A. and WILSON, R. P. Effect of orotic acid on the lipid and soluble nucleotide concentrations in avian liver. Biochim. Biophys. Acta, 187: 266-268, 1969.

15. KRUSKI, A. W. and NARAYAN, K. A. Effect of orotic acid and cholesterol on the synthesis and composition of chicken (Gallus domesticus) serum lipoprotein. Int. J. Biochem., 7: 635-638, 1976.

16. ROBINSON, J. L. Bovine milk orotic acid: Variability and significance for human nutrition. J. Dairy Sci., 63: 865-871, 1980.

17. WINDMUELLER, H. G., MCDANIEL, E. G. and SPAETH, A. Orotic acid-induced fatty liver. Metabolic studies in conventional and germ-free rats. Arch. Biochem. Biophys., 109: 13-19, 1965.

18. ADACHI, T., TANIMURA, A. and ASAHINA, M. A colorimetric determination of orotic acid. J. Vitamin, 9: 217-226, 1963.

19. FOLCH, J., LEES, M. and SLOANE-STANLEY, G. H. A simple method for the isolation and purification of total lipids from animal tissues. J. Biochem., 226: 497-509, 1957.

20. STEEL, R. G. D. and TORRIE, J. H. Principles and Procedures of Statistics, McGraw-Hill, New York, 1960.

21. WINDHOLZ, M., BUDAVARI, S., STROUMTSOS, L. Y. and FERTIG, M. N. The Merck Index, Ninth Edition, Merck and Co., New Jersey, 1976, p. 20.

22. CLIFFORD, A. J. and STORY, D. L. Levels of purines in foods and their metabolic effects in rats. J. Nutr., 106: 435-442, 1976.

23. MILNER, J. A. Mechanism for fatty liver induction in rats fed arginine deficient diets. J. Nutr., 109: 663-670, 1979.

NUTRITION RESEARCH, Vol. 1, pp. 261-266, 1981
0271-5317/81/030261-06$02.00/0 Printed in the USA.
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DEPLETION OF HEART AND SKELETAL MUSCLE CARNITINE IN THE NORMAL RAT BY PERITONEAL DIALYSIS

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ABSTRACT

Serum carnitine values are markedly reduced during dialysis in patients with end-stage renal disease. In an effort to obtain more evidence for dialysis induced carnitine depletion in tissue an animal model was developed. It was found that a relatively short term peritoneal dialysis treatment in the normal rat led to a 50% decrease in serum carnitine and a 50% reduction of carnitine in the heart and skeletal muscle. The concentration of carnitine in liver did not change. The results indicate that this model may be suitable for determining specific effects of carnitine depletion in heart and skeletal muscle as well as studying the effects of dialysis under various experimental conditions.
carnitine, heart, skeletal muscle, liver, dialysis

INTRODUCTION

Carnitine (β-hydroxy-γ-trimethylamino butyrate) is recognized as an important nutrient acting as a co-factor in the β oxidation of fatty acids and possibly in the metabolism of branch chain amino acids (1). Because carnitine is readily available in food and can be synthesized de novo in the liver and/or kidney from methionine and protein bound trimethyllysine, a specific deficiency state has been extremely difficult to produce by dietary means in the experimental animal.

There has been a growing appreciation of the fact that carnitine, which is a low molecular weight substance not bound to larger macromolecules, can be easily removed from the serum by dialysis. Recently, a number of reports have indicated that in patients with chronic renal disease, dialysis produces a sharp decline in serum carnitine which can be quantitatively accounted for by its accumulation in the dialysate (2-5). Furthermore, in some subjects a marked reduction in skeletal muscle carnitine has also been noted (2). In an effort to apply results of these clinical studies to more detailed metabolic protocols in the experimental animal, it became apparent that hemodialysis in the rat was not feasible. We, therefore, considered and successfully devised a simple, workable peritoneal dialysis model. In this communication we provide evidence for the production of a carnitine deficiency in heart and skeletal muscle in the otherwise normal rat after only two successive eight-hour periods of peritoneal dialysis 24 hours apart.

MATERIALS AND METHODS

Sprague-Dawley male rats weighing approximately 200 grams were fed an ad libitum diet of Purina Rat Chow which contained an average of 60 nmol/gram carnitine. The dialysis cannula was made by cutting a number eight French infant feeding catheter to a 16 cm length and inserting eight 1-2 mm pores in the

distal 3 cm. Ether anesthesia was used to prepare the rat for cannula installation. Two incisions were made through the skin, one of 0.5 cm at the midscapula region and one of 2-3 cm at the ventral abdominal region. A blunt dissecting probe was threaded through from the abdominal to the scapular incision. The distal end of the catheter was affixed and the catheter pulled under the skin layer. A 2-3 mm entry hole was placed through the abdominal muscle layers 2 cm to the left of the midline, and the distal end of the catheter was inserted. All cannula pores (approximately 5 cm cannula) were within the peritoneal cavity, and an anchoring stitch was placed in the muscle layer around the cannula entry point. An anchoring stitch was also tied around the cannula at the skin entry point in the midscapula region. The abdominal skin incision was closed with 4-0 silk.

The dialysis procedure involved the inflow of a total of 600 ml of dialysate fluid at 38°C each day. Fifteen cycles of 40 ml each of peritoneal dialysate were injected and removed every 30 minutes. The total dialysate solution removed yielded 600 $\pm$ 12.5 ml at the end of each eight hour procedure. The dialysis fluid which was a preparation for clinical use contained in one liter: 130 mEq sodium, 3.5 mEq calcium, 1.0 mEq magnesium, 100 mEq chloride, 34.5 mEq acetate and 25 grams dextrose.

Blood samples were drawn from the tail vein at two hour intervals during dialysis and at four hour intervals post-dialysis. At the end of the second dialysis the animals were anesthetized with ether, weighed and exsanguinated by heart puncture. Nondialyzed cannulated rats served as controls. Liver, skeletal muscle and heart muscle were immediately removed, frozen in liquid nitrogen and stored at -100° until extracted and analyzed. Serum samples obtained from the cardiac blood or tail vein were also stored under identical conditions. The frozen tissue was finally powdered with a mortar and pestle, and the powder homogenized in 2 ml of 2.5 M $HClO_4$. The homogenate was then centrifuged at 0-4° for ten minutes at 5,000 g. The supernatant fluid was removed and the solid residue washed two times with 1 ml H_2O, mixed and centrifuged as above. All of the supernatants were pooled. The residue was dried in a 60° drying oven to a constant weight which was the dry weight of the tissues analyzed. Approximately 1 ml of 2.2 M KOH was added to the pooled supernates and the solution neutralized to pH 7 - 7.2. The contents of the tubes were mixed, allowed to stand for 30 minutes at 0-4°, and the $HClO_4$ precipitate removed by centrifugation. To obtain short chain acid soluble acylcarnitine, 0.05 ml aliquots of the tissue supernatants or serum were added to 0.1 ml of a 1.0 M tris base and 0.05 ml of 0.4 M KOH and allowed to stand at 30° for one hour to hydrolyze the acylcarnitine. Under normal conditions free plus acid soluble short chain acylcarnitine constitutes 90% of the total in both serum and muscle (5,6). Free carnitine was then determined by the radioisotopic enzymatic method of McGarry and Foster (7).

RESULTS

A protocol was developed to reduce the rat serum carnitine levels to 40-60% of normal or approximately that which was observed in patients on maintenance dialysis (5). It was established that two consecutive days of dialysis were required to achieve this condition.

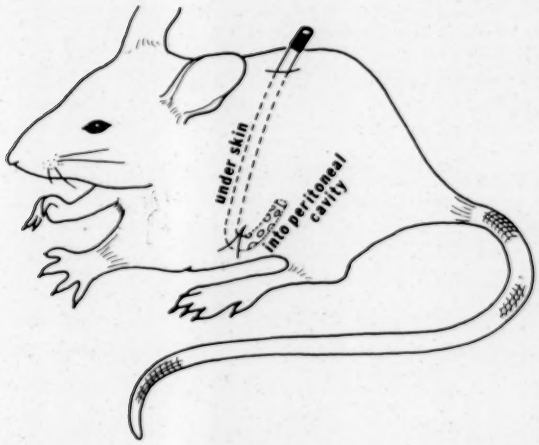

FIG. 1

Diagram of the Rat Prepared for Peritoneal Dialysis

Figure 1 is a diagram of the rat model with a catheter inserted in place. The animals were allowed free access to food and water prior to and between the experimental dialysis periods, and there were no weight changes. The success of the procedure required keeping the cannula in place over a three - four day period while permitting the rat to eat and drink normally, maintaining an adequate state of health during this procedure and preventing tissue infiltration from plugging the cannula pores. Whereas under these conditions the overall procedure was relatively simple, it is appreciated that a similar experimental approach in the uremic animal would be much more complicated.

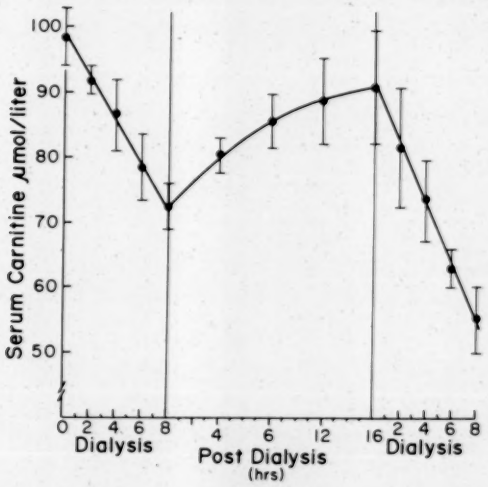

FIG. 2

Changes in Serum Carnitine During Peritoneal Dialysis in the Rat. The experiments were carried out as described under Materials and Methods. Each point represents the average value and S.E.M. of nine animals.

During the first eight-hour dialysis period the serum carnitine dropped to approximately 70% of control values, whereas in the sixteen-hour interim between dialysis the levels gradually increased again to near the control values (Fig. 2). During the second eight hours of dialysis the decline in serum carnitine was even greater, the levels decreasing to approximately 50% of the control values. These findings are very similar to those obtained in patients with end-stage renal disease undergoing maintenance hemodialysis therapy (5). It was necessary to use a total of 600 ml during each dialysis to obtain these results. Similar experiments using 300 ml of fluid per dialysis reduced serum carnitine values to only 65% of control by the second day.

TABLE 1

Effect of Peritoneal Dialysis on Serum, Heart and Skeletal Muscle Carnitine Concentrations

Experiments were carried out as described in Materials and Methods. Animals were sacrificed after the second dialysis period.

EXPERIMENTAL GROUP	n	WT.	CARNITINE (free + acid soluble)			
			Serum	Liver	Heart Muscle	Skeletal Muscle
			µmol/liter		µmol/g dry wt.	
Control	9	208 ± 8.8*	86.9 ± 7.6	0.87 ± 0.11	5.94 ± 0.28	4.67 ± 0.21
Dialyzed	9	205 ± 6.7	42.2 ± 6.4+	0.77 ± 0.06	3.43 ± 0.90+	2.12 ± 0.39+

*S.E.M.

+$p < 0.001$ against control

Liver, heart and skeletal muscle, tissues which all contain a high content of carnitine (8), were sampled for analysis at the completion of the second dialysis period when the serum concentration reached 50% of control values. At this time there were significant decreases in the concentrations of carnitine in heart and skeletal muscle (Table 1). The values represent the sum of free and acid soluble acylcarnitine which accounts for over 90% of the total carnitine in tissue (6). By contrast, the concentration of carnitine in liver was still similar to that of the control animals. The experiments were not carried further to determine whether the concentration of carnitine in liver could also be reduced after a longer interval of dialysis treatment.

DISCUSSION

Deficiencies of carnitine in heart and skeletal muscle (9,10) and of the enzyme carnitine palmitoyl transferase (11) are now recognized as specific genetic diseases in man producing a characteristic clinical neuromuscular syndrome which can be traced to impaired fatty acid oxidation. A systemic and often lethal carnitine deficiency state is also characterized by severe depletion of carnitine in the liver and associated with hypoglycemia and neurological symptoms (12). The administration of carnitine to patients with primary Type IV hyperlipoproteinemia (13) as well as those with hypertriglyceridemia secondary to chronic renal disease and hemodialysis therapy (14,15) was reported to significantly decrease their circulating triglycerides. With the rapid accumulation of information indicating the importance of carnitine in health and disease, an experimental animal model offers considerable advantages for studying the abnormal metabolism related to carnitine deficiency.

In the rat the liver is the major site of carnitine biosynthesis, and this organ serves as the source of carnitine for other tissues including heart and

skeletal muscle (16). Under the experimental conditions used in this study there was no reason to expect that carnitine synthesis would be impaired. The animals were allowed continual access to food which contained, in addition to the usual required nutrients, adequate amounts of carnitine, and they were noted to eat normally. The animals all appeared well and active during the study, and there was no indication that dialysis created any untoward effects other than removing low molecular weight solutes. Yet even under these relatively mild conditions the dialysis treatment itself was able to reduce carnitine in the heart and skeletal muscle approximately 50% within a very short time. The inability to deplete the liver of carnitine under these circumstances might be expected since this organ serves as a major site for carnitine biosynthesis in the rat.

The present investigation indicates that a simple rat model system can be devised to rapidly deplete the animal of carnitine in heart and skeletal muscle. Biochemically at least the model resembles the myopathic carnitine deficiency state in man (17). The results of these experiments clearly indicate that dialysis even over a short time interval is potentially able to deplete body stores of small molecular weight substances and supports the report that tissue carnitine depletion occurs clinically (2). Moreover, dialysis seems to be effective independently of the renal status of the subject. This experimental model has the advantage of manipulating the concentration of small molecular weight substances by including or omitting them in the dialysate. It is, therefore, now feasible to carry out a variety of *in vivo* and *in vitro* investigations which should lead to a better understanding of the biochemistry, pathophysiology and clinical consequences of potential deficiency states produced by dialysis. In addition, it may now be possible to study specific biochemical and physiological abnormalities of a myopathic carnitine deficiency state in the experimental animal.

ACKNOWLEDGEMENT

We appreciate the excellent technical assistance of Mr. William Patterson in developing the rat peritoneal dialysis system. This work was supported in part by U.S.P.H.S. Grant GM-14033 and the Wisconsin affiliate of the American Diabetes Association.

REFERENCES

1. MITCHELL, M.E. Carnitine metabolism in human subjects. 1. Normal metabolism. Am.J.Clin.Nutr.,31: 293-306, 1978.

2. BOHMER, T., BERGREM, H. and EIKLID, K. Carnitine deficiency induced during intermittent hemodialysis for renal failure. Lancet, 2: 126-128, 1978.

3. BIZZI, A., MINGARDI, G., CODEGONI, A.M., MECCA, G. and GARATTINI, S. Accelerated recovery of post-dialysis plasma carnitine fall by oral carnitine. Biomedicine, 29: 183-184, 1978.

4. MINGARDI, G., BIZZI, A., CINI, M., LICINE, R., MECCA, G. and GARATTINI, S. Carnitine balance in hemodialyzed patients. Clin. Nephrol., 13: 269-270, 1980.

5. BARTEL, L.L., HUSSEY, J.L. and SHRAGO, E. Perturbation of serum carnitine levels in human adults by chronic renal disease and hemodialysis therapy. Am. J. Clin. Nutr. (in press).

6. IDELL-WENGER, J.A., GROTYAHANN, L.W. and NEELEY, J.R. Co-enzyme A and carnitine distribution in normal and ischemic hearts. J. Biol. Chem., 253: 4310-4318, 1978.

7. MC GARRY, J.D. and FOSTER, D.W. An improved and simplified radioisotopic assay for the determination of free and esterified carnitine. J. Lipid Res. 17: 277-281, 1976.

8. MITCHELL, M.E. Carnitine metabolism in human subjects. 3. Metabolism in disease. Am.J.Clin.Nutr., 31: 645-659, 1978.

9. ENGEL, A. and ANGELINI, C. Carnitine deficiency in human skeletal muscle with associated lipid storage myopathy; a new syndrome. Science, 179: 899-902, 1973.

10. ANGELINI, C. Carnitine deficiency. Lancet, 2: 554, 1975.

11. DI MAURO, S. and DI MAURO, P. Muscle palmitoyl carnitine transferase deficiency in myoglobinuria. Science, 182: 929-931, 1973.

12. WARE, A.J., BURTON, W.C., MC GARRY, J.D., MARX, J.F. and WEINBERG, A.G. Systemic carnitine deficiency: Report of a fatal case with multi-systemic manifestations. J. Ped., 93: 959-964, 1978.

13. MAEBASHI, M., KAWAMURA, N., SATO, M., IMAMURA, A. AND YOSHINGA, K. Lipid lowering effects of carnitine in patients with Type IV hyperlipoproteinemia. Lancet, 2: 805-807, 1978.

14. GUARNIERI, G., RANIERI, F., TOIGO, G., VASILE, A., CIMAN, M., RIZZOLI, V., MARACCHIELLO, M. and CAMPANACCI, L. Lipid lowering effect of carnitine in chronically uremic patients with maintenance hemodialysis. Am. J. Clin. Nutr., 33: 1489-1492, 1980.

15. LACOUR, B., CHANARD, J., HAGUET, M., BASILE, C. ASSAN, R., DI GUILIO, S., CIANCIONI, C., LEBKIRE, B., DRUEKE, T. and FUNCK-BRENTANO, J.L. Carnitine improves lipid anomalies in hemodialysis patients. Lancet, 2: 763-764, 1980.

16. TAMPHAICHITR, V. and BROQUIST, H.P. Site of carnitine biosynthesis in the rat. J. Nutr., 104: 1669-1673, 1974.

17. DI MAURO, S., TREVISAN, C. and HAYS, A. Disorders of lipid metabolism in muscle. Muscle and Nerve, 3: 369-388, 1980.

NUTRITION RESEARCH, Vol. 1, pp. 267-276, 1981
0271-5317/81/030267-10$02.00/0 Printed in the USA.
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SPECIFIC EFFECTS OF PROTEIN DEPRIVATION ON THYMUS AND SPLEEN; RATES OF PROTEIN SYNTHESIS IN LYMPHOCYTES DERIVED FROM THYMUS, SPLEEN AND BONE MARROW

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ABSTRACT

Young adult male rats (body weight 90 g) were fed for 6 days a low protein (3% casein) diet and compared with rats fed a diet optimal in protein (20% casein). To study the dietary effects of protein, but not energy deficiency, intake of energy per unit body weight was kept constant. Wet weights and DNA content of thymus and spleen organs decreased in the low protein diet group. The in vitro incorporation of ^{14}C-leucine into protein by isolated ribosomes was diminished in thymus by 22% and in spleen by 33% per mg of ribosomal RNA, and per mg of DNA by 43% in thymus and 48% in spleen. Lymphocytes were separated by Ficoll gradients. As compared with controls lymphocyte number from thymus decreased by 90%, that of spleen by 43% and of bone marrow by 20%. The rates of protein synthesis were significantly reduced after protein restriction in lymphocytes of the 3 organs. The strongest effects on the rates of protein synthesis were observed in bone marrow. Reduction in lymphocytes recovered was most pronounced in thymus. The results indicate that malnutrition by protein, but not energy deficiency, elicites specific effects on the 3 lymphocyte producing or containing organs studied.
KEY WORDS: Protein restriction • thymus • spleen • bone marrow • lymphocytes • protein synthesis

INTRODUCTION

A restricted intake of dietary proteins leads to a reduction in the capacity of protein synthesis in vitro by ribosomes of thymus, spleen and bone marrow (1). Ingestion by rats of diets low in protein content is less than that of diets optimal in protein content (2). The differences in energy intake are without effect on observed protein diet dependent changes in protein synthesis in tissues such as liver and skeletal muscle (2). Thymus is a sensitive monitor of both protein malnutrition and starvation (3, 4). Reduction in energy intake may accentuate the metabolic changes observed previously with a protein restricted diet (1).

Thymus, spleen and bone marrow contain lymphoid cells which participate directly or cooparatively in immune responses. So far no effect of short-term protein starvation on isolated lymphoid cells has been reported. Alterations of cell-mediated immunity in humans are observed during protein-energy malnutrition (5, 6). As previous studies on the effects of protein restriction were concerned with the whole cell population of the organ rather than with a specific cell type (1), it is important for a better understanding of metabolic events to examine specific effects of short-term protein deficiency on a selected cell population.

In the present study a diet low in protein was compared at equal energy

intake per unit body weight with one optimal in protein content. The capacity for protein synthesis was studied in ribosomes obtained from thymus and spleen. Lymphoid cells were isolated in order to determine whether differences in the rates of protein synthesis resulting from protein but not energy malnutrition are organ and cell specific.

MATERIALS AND METHODS

Animals. Male 25-day old Sprague-Dawley rats (Sprague-Dawley, Inc., Madison, WI) initially weighing 80 g were individually housed in metal cages, under a 12:12 hour light-dark cycle at 22° ± 1 and a relative humidity of 50%. After a prefeeding period of 3 days during which a 10% casein (Vitamin-free casein, pH 5.8, from United States Biochemical Corporation, Cleveland, OH) diet was fed, the rats weighing 93.8 ± 1.3 g were randomly divided into two groups. They were fed an isocaloric diet containing either 20% or 3% of casein supplemented with 3 g of methionine/kg of protein. The rats were pairfed with a constant energy intake per unit body weight. Details of the diets have been given previously (1). Rats on the 3% protein containing diet were fed ad libitum. The food consumed per day was measured at the end of the light period. The corresponding amount of the 20% protein diet plus an additional 1 g of food per 10 g increase in body weight was fed to the other group of rats (7). These rats began with 10 g food during the first day. Water was provided ad libitum. After consuming the 20% or 3% protein diet for 6 days the rats were killed at 08.00 hours, without previous starvation, by dislocation of the spine.

Preparation of ribosomes and cell sap. Preparation of ribosomes from thymus and spleen and of cell sap from liver was done as described previously (1, 8).

For studies of polyribosomal pattern suspensions of isolated ribosomes corresponding to 50 µg of RNA were layered onto gradients of 0.4 to 1.3 M sucrose in 0.035 M Tris-acetic acid pH 6 measured at 25°, 0.025 M KCl, 0.040 M NH_4Cl, and 0.005 M $MgSO_4$. Centrifugation was in an ultracentrifuge (Beckman L2-65B; Beckman Instruments, Inc., Palo Alto, CA) for 55 minutes at 149 000 x *g* in a SW 50.1 rotor. The absorbance pattern of the gradient was measured at 260 nm.

Amino acid incorporation by ribosomes. The incubation mixture contained phosphocreatine 25 mM, creatine phosphokinase 0.5 units, ATP 1 mM, GTP 0.1 mM, leucine-free amino acid mixture 1.4 µg total (8), ^{14}C-l-leucine 0.05 µCi of the L-form, sucrose 0.25 M, Tris-HCl pH 7.8 measured at 25° 0.035 M, KCl 0.025 M, NH_4Cl 0.040 M, and $MgSO_4$ 0.005 M, Sephadex-treated liver cell sap (0.5 mg of protein), and increasing amounts of ribosomes (15 - 50 µg of ribosomal RNA) (1). The final incubation volume was 120 µl. After incubation at 35° for 15 minutes proteins were precipitated in trichloroacetic acid, extracted with hot trichloroacetic acid and radioactivity was measured in a scintillation spectrometer (Intertechnique Liquid Scintillation Spectrometer, Model SL 30, Plaisir, France) at 60% efficiency.

Isolation and incubation of lymphoid cells. Minimum Essential Medium with Earl's Salts, EMEM (Flow Laboratories Svenska AB, Solna, Sweden) was used throughout. Thymus and spleen were transferred to the medium. Cells were squeezed out gently at 20° with a forceps. The connective tissue was then removed. Bone marrow cells were recovered from the two femurs by injecting under pressure medium into the bone with a syringe. The individual cell suspensions in 4 ml of medium were layered onto 3 ml of Ficoll-Paque (Pharmacia Fine Chemicals, Uppsala, Sweden) as described in the Pharmacia Ficoll-Paque Manual. The procedure for isolation of lymphocytes from blood described in the Pharmacia Manual was followed. The isolated lymphocytes were suspended in medium at a concentration of 2 x 10^6 to 5 x 10^6 cells per ml. Viability was 95% as determined by tryphan blue exclusion method. Purity of the cells was confirmed using May-Grünwald Giemsa and Haematoxylin (acc. to Heidenhain)

staining (9). Contamination by cells other than lymphocytes was less than 1%. The cell suspension was kept at 37° for 15 minutes before addition of 1.9 µCi/ml of the ^{14}C-amino acid mixture. At the times indicated (see Fig. 2) duplicate samples of 100 µl of incubation suspension were withdrawn and pipetted onto filterpaper discs. These were placed into ice-cold trichloroacetic acid, extracted with hot trichloroacetic acid and radioactivity was determined. Uptake of radioactive amino acids by the cells was determined by incubating the cells as above. After incubation the cells were washed twice in medium. The cells were suspended in 1.5 ml of medium and 100 µl samples withdrawn. One set was dried and total radioactivity determined, the other set extracted with hot trichloroacetic acid and radioactivity of the proteins determined. This value was subtracted from the total radioactivity to give the activity of the free amino acids taken up by the cells, after differences in counting efficiency had been corrected.

Analyses. RNA was extracted from the ribosomes in 0.4 M $HClO_4$, and the absorbance at 260 nm measured. RNA was calculated on the basis of 34.2 optical density units at 260 nm/mg of RNA. DNA was analysed by a fluorometric method (10) using salmon DNA as a standard.

Statistical evaluation. The results were evaluated using the Student's t-test (11). The results are presented as the mean values ±SEM.

Materials. Chemicals were of analytical grade whenever possible. The sources were given in detail previously (1, 8). In addition, L-(U-^{14}C)amino acid mixture CFB 104, specific activity 58 mCi/mAtom carbon was obtained from Amersham International Ltd., Amersham, UK.

RESULTS

The daily intake of food restricted in proteins decreased during the 6 day-feeding period (Table 1).

TABLE 1
Daily Food Intake of the Diet low in Protein

Day on diet	Food intake KJ	Food intake g
1	237±12[a]	13.4±0.7[a]
2	224± 9[a]	12.7±0.5[a]
3	207±11[a,b]	11.7±0.6[a,b]
4	186± 9[b]	10.5±0.5[b]
5	180±11[b]	10.2±0.6[b]
6	127± 7	7.2±0.4

The results are the mean values ±SEM of 18 rats. Results with the same superscript letter are not significantly different. Significance, $P<0.01$.

The low intake on day 6 shows that 70% of the daily food consumption took place during the dark hours. As in growing animals basic metabolism increases with body size and weight, this was taken into account by increasing the daily food ration of the rats fed the 20% protein diet with 1 g of food for every 10 g increase in body weight. Protein restriction resulted in a small decrease in body weight, while the pairfed animals increased in body weight (Table 2). Organ wet weights were also affected by the reduced protein intake being significantly diminished in both total wet weight and relative to a standard body weight (Table 2). Cell number as determined by DNA content was reduced and thus partially responsible for the decrease in wet weight (Table 3). Similarly the

number of ribosomes measured as ribosomal RNA per total wet weight was low, while relative to DNA the ribosome concentration remained unchanged in spleen and showed no significant decrease in thymus.

TABLE 2
Change in Body Weight and Organ Wet Weight after Protein Restriction

Diet	Change in body weight per day	Thymus (g) Total	Thymus (g) Per 100 g body weight	Spleen (g) Total	Spleen (g) Per 100 g body weight
20% protein	+6.6±0.2	0.49±0.02	0.37±0.01	0.47±0.02	0.35±0.01
3% protein	-0.7±0.1	0.19±0.01	0.21±0.01	0.19±0.01	0.21±0.01

The results are the mean values ±SEM of 20 rats per dietary group. Body weight at the start of the experimental diet was 93.8±1.3 g. All differences in results between dietary groups are significant, P<0.001.

TABLE 3
Effect of Protein Restriction on DNA and Ribosomal RNA Content

Organ	Diet	DNA (mg) Total	DNA (mg) Per g tissue wet weight	rRNA (mg) Total	rRNA (mg) Per g tissue wet weight	Ratio rRNA:DNA
Thymus	20% protein	15.30±1.61	33.06±3.31	1.13±0.08	2.39±0.09	0.07±0.01
	3% protein	6.64±0.58	34.93±3.29	0.37±0.03	1.94±0.13	0.06±0.01
	Significance	P<0.001	NS	P<0.001	P<0.02	NS
Spleen	20% protein	10.24±0.43	22.24±1.37	0.95±0.07	1.95±0.10	0.09±0.01
	3% protein	3.90±0.26	19.87±1.04	0.28±0.02	1.56±0.10	0.09±0.01
	Significance	P<0.001	NS	P<0.001	P<0.02	NS

The results are the mean values ±SEM of 8 (thymus) and 12 (spleen) rats per dietary group. NS, not significant.

TABLE 4
Amino Acid Incorporating Activity of Ribosomes after Protein Restriction

Organ	Diet	pmol leucine incorporated into protein per mg ribosomal RNA	Total ribosomal RNA	mg DNA
Thymus	20% protein	1,116.3±19.9	1,280.5±88.6	90.1±9.2
	3% protein	867.1±20.4	326.0±26.9	51.5±5.6
Spleen	20% protein	694.7±17.8	638.6±69.0	57.3±6.5
	3% protein	463.7±12.7	122.1±12.6	30.0±3.4

The results are the mean values ±SEM of 8 independent experiments per dietary group and 6 determinations per experiment. All differences in results between dietary groups are significant, P<0.001.

Ribosomes were isolated from thymus and spleen and their capacity for amino acid incorporation into protein measured (Table 4). ^{14}C-leucine was used as radioactive precursor. Activity expressed as pmol leucine incorporated into protein was significantly reduced after protein restriction both per mg of ribosomal RNA, total RNA and mg of DNA. Results per unit of DNA indicate a diminished capacity for protein synthesis per cell.

The non-ionic detergent Triton X-100 has previously been used to break up thymic cells effectively during homogenization (1). When Triton X-100 was added to total homogenates of spleen a reduction in protein synthesis by the isolated ribosomes was observed. This was most pronounced in preparations obtained from rats fed the 20% protein diet. Polyribosomal profiles showed an increase in monoribosomes relative to polyribosomes when Triton was present during homogenization of the organ (compare Fig. 1a, b with c, d). Release or activation of RNase was responsible for the change in polyribosomal pattern. The effect was less in rats fed the low protein diet (Fig. 1b and d). In the experiments presented in Table 4 the spleens were homogenized in the absence of detergent.

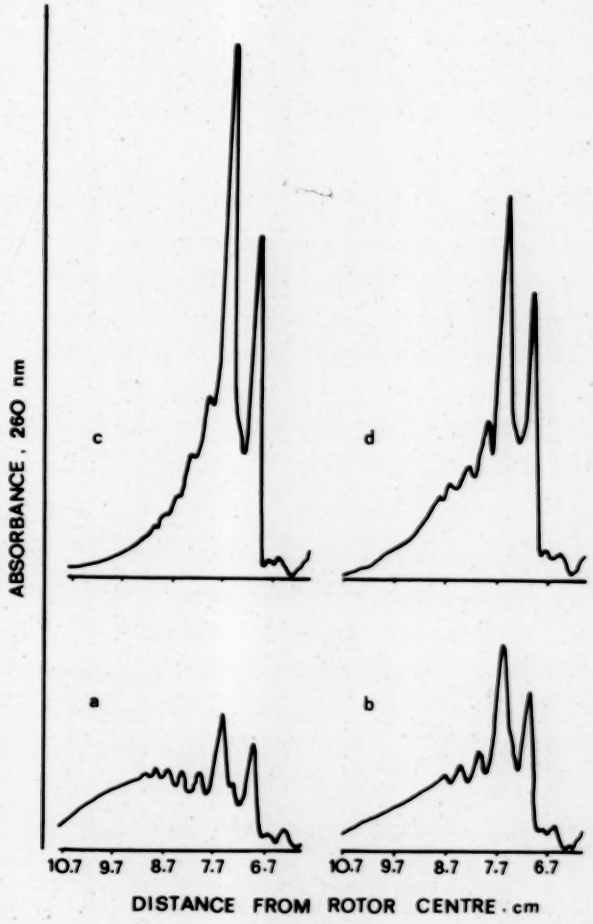

FIG. 1
Effect of activated RNase on polyribosomal pattern of spleen ribosomes. Polyribosomes of rats fed, 20% protein (a and c), 3% protein (b and d). Spleen was homogenized in the absence (a and b) or the presence (c and d) of 0.25% Triton X-100.

Lymphocytes of thymus, spleen and bone marrow were isolated to study the specific effect of protein malnutrition on this particular cell type rather than on the whole cell population of the organs (Fig. 2). A significantly diminished rate of protein synthesis was observed in lymphocytes derived from all 3 organs of protein restricted rats. The differences between the dietary groups were most pronounced in spleen and bone marrow. The rates of protein synthesis in thymic and spleen lymphocytes of rats fed 20% protein were similar. Bone marrow lymphocytes showed an increased rate indicating the high demand for new proteins in the proliferating lymphoid cell producing organ.

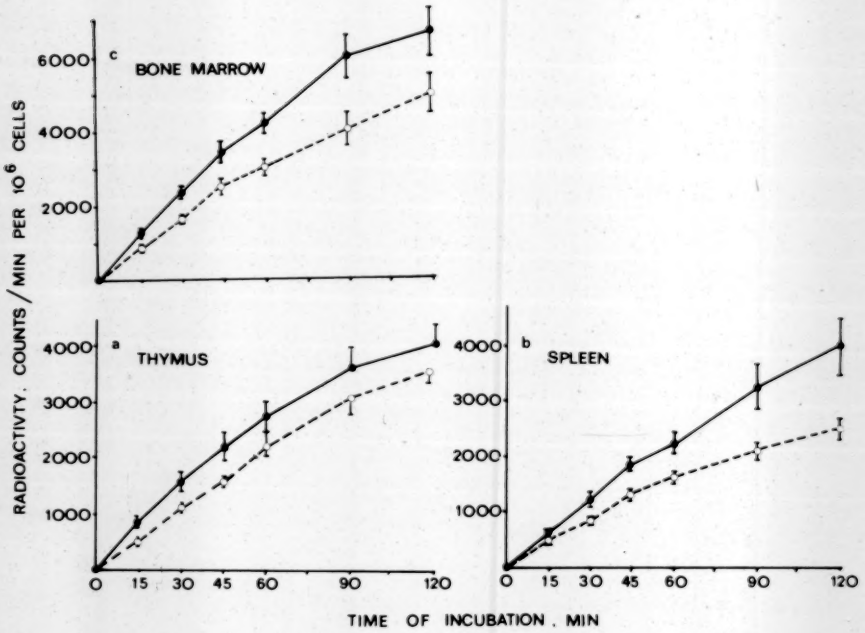

FIG. 2
Rates of protein synthesis by isolated lymphocytes recovered from a) thymi, b) spleens, c) bone marrow. Rats fed 20% protein ●——●, or 3% protein o-----o. The results are the mean values of 8 (thymus and spleen) and 12 (bone marrow) independent experiments ±SEM as indicated by vertical bars. Note differences in scale of ordinates.

Cell yield was significantly diminished after protein restriction (Table 5). In thymus, DNA content was reduced to 43% of the control value (Table 3), but the yield of lymphocytes was reduced to 10% indicating a marked loss of lymphocytes during involution of the organ. The DNA content of spleen was reduced to 38% and lymphocyte yield to 57%. Changes in spleen showed a proportional loss of lymphocytes. Bone marrow cell yield was least affected by protein malnutrition.

TABLE 5
Recovery of Lymphocytes after Separation on Ficoll-Paque

Organ	Diet	Cell counts x 10^{-6}
Thymus	20% protein	1,161.00±137.12
	3% protein	117.26± 24.06
	Significance	P<0.001
Spleen	20% protein	39.55± 1.89
	3% protein	22.57± 2.90
	Significance	P<0.001
Bone marrow	20% protein	12.75± 0.89
	3% protein	10.22± 0.80
	Significance	P<0.05

The results are the mean values ±SEM of 8(thymus and spleen) and 12 (bone marrow) independent experiments per dietary group.

The differences in the rate of protein synthesis observed in lymphocytes were not a function of amino acid uptake by the cells. Concentration of radioactive amino acids in the cells was almost maximal after 20 min at 35°. The percentage differences in uptake were minimal between lymphocytes of the 2 dietary groups. After 15 min of incubation differences in uptake amounted to 1.2 ± 0.1%.

DISCUSSION

The specific effect of malnutrition with protein deficiency on lymphoid cell producing or containing organs was demonstrated. Energy intake per unit body weight was kept constant and was thus not responsible for the differences observed. Involution of thymus was associated with a loss of lymphoid cells. As viewed by electron microscopy, the thymus is a solid epithelial organ (12), in which lymphocytes are surrounded by the epithelial cells. A decrease in number of lymphocytes may indicate a reduced frequency of mitosis (13, 14). Circulating thymus-derived lymphocytes are reduced during protein-energy malnutrition (5, 15), and a release into the blood of lymphocytes from the thymus as a result of nutritional imbalance seems therefore unlikely. Wet weight and lymphocyte concentration were also reduced in spleen. The nutritional effect was not limited to lymphoid cells only but was evident in the whole organ. The yield of lymphocytes from bone marrow was least affected. The marked reduction of the rate of protein synthesis showed the specific effect of protein restriction on these cells. Bone marrow is the major site of production of lymphoid and haematopoietic cells. Previously a reduction in protein synthesis by isolated ribosomes from the whole cell population was shown when rats were fed ad libitum a 3% as compared with a 20% protein diet (1). In the present experiments with controlled energy intake a specific effect of protein malnutrition on isolated bone marrow lymphocytes was demonstrated.

Activity of ribosomes isolated from thymus and spleen confirmed previous results of a reduced capacity for protein synthesis (1). Active RNase was not responsible for the decrease as shown by the polyribosomal pattern of spleen (Fig. 1a and b) and of thymus (1). RNase will hydrolyze mRNA and thus break up polyribosomes to monoribosomes. An activation of RNase was observed when spleen

was homogenized in detergent, but RNase activity was low rather than high during protein restriction. RNase found in spleen must be part of the phagocytic cells containing hydrolytic enzyme activities.

Maximal daily energy intake has to be decreased by more than 50% before an effect on protein synthesis in skeletal muscle and liver is observed (2). Intake of 176 KJ/day by rats of more than 38 days of age (more than 110 g body weight) is 70% that of the maximal intake (2). The results obtained here by the diet low in protein was therefore a specific effect of protein restriction and not of energy starvation. Energy intake was more than 176 KJ per 24 h feeding period.

Rats fed ad libitum a diet optimal in proteins increased in body weight by more than 7.5 g/day (1, 2, 16, 17). With the constant energy intake per unit body weight the increase in weight was 6.6 g/day and the wet weights of thymus were less than in rats fed ad libitum a diet optimal in proteins (1). This agrees with early findings that the size of thymus is subject to changes by both protein and energy malnutrition (3, 4).

While extreme nutrient deficiencies cause vast changes in immune functions of the body, mild reduction in energy and protein intake is associated with an increased life span in certain inbred mice (18, 19). Severe protein-energy malnutrition as well as deficiencies of many specific nutrients impair antibody production in vivo (20), reduce the number of circulating lymphocytes (5, 15), and modulate responses to thymus-dependent antigens, cell functions (15), and lymphocyte transformations (21). Levels of thymic hormone activities in the blood are low (5). If the hormone is involved in maturation this may lead to a retardation in differentiation of lymphocytes present in thymus (5).

Recent studies have demonstrated that subsets of lymphocytes are involved in a complex series of interactions that regulate immune responses (22, 23). No differentiation between lymphocyte subsets has been made here. The experiments described add to the information on biological properties of lymphoid cells. Short-term malnutrition with protein but not energy deficiency was specifically expressed as a low capacity for protein synthesis and reduced yield of lymphoid cells obtained from thymus, spleen and bone marrow. Among the 3 organs, reduction in recovery of lymphoid cells was most pronounced in thymus, while the rates of protein synthesis were most strongly affected in lymphocytes isolated from bone marrow.

ACKNOWLEDGEMENT

The skilled technical assistance of Mrs. Christina Olgar is gratefully acknowledged.

REFERENCES

1. VON DER DECKEN, A., LUND, B. and O'TOOLE, C. Effect of a low protein diet on in vitro protein synthesis in thymus, spleen, and bone marrow in young adult rats. J. Nutr. 108:1274-1280, 1978.

2. OMSTEDT, P. and VON DER DECKEN, A. Dietary amino acids: effect of depletion and recovery on protein synthesis in vitro in rat skeletal muscle and liver. Br. J. Nutr. 31:67-76, 1974.

3. SIMON, J. A Physiological Essay on the Thymus Gland. H. Renshaw, London, 1845.

4. HAMMAR, J.A. Fünfzig Jahre Thymusforschung. Ergebnisse der Anatomie und Entwickelungsgeschichte. 19:1-274, 1909.

5. CHANDRA, R.K. Cell-mediated immunity in nutritional imbalance. Fed. Proc. 39:3088-3092, 1980.

6. GROSS, R.L. & NEWBERNE, P.M. Role of nutrition in immunologic function. Physiol. Rev. 60:188-302, 1980.

7. BROCK, N., BARTLING, H. and WILK, W. Nutrition of Laboratory Animals. Ed. Editio Cantor, Aulendorf/Württ., Germany, 1961.

8. VON DER DECKEN, A. Modification of the in vitro amino acid incorporation capacity of rat liver after in vitro and in vivo treatments. Eur. J. Biochem. 4:87-94, 1968.

9. ROMEIS, B. Mikroskopische Technik. Leibniz Verlag, München, 1948.

10. SETARO, F. and MORLEY, C.G.D. A modified fluorometric method for the determination of microgram quantities of DNA from cell or tissue cultures. Analyt. Biochem. 71:313-317, 1976.

11. SNEDECOR, G.W. Statistical Methods (6th Edition). Iowa State University Press, Ames, IA, 1967.

12. CLARK, S.L. Jr. The thymus in mice of strain 129/J studied with the electron microscope. Amer. J. Anat. 112:1-9, 1963.

13. KENNEY, M.A., RODERBRUCK, C.E., ARNICH, L. and PIEDAD, F. Effect of protein deficiency on the spleen and antibody response in rats. J. Nutr. 95:173-178, 1968.

14. ASCHKENASY, A. Comparative effects of a protein deprived diet on the weight, lymphocyte content and the number of DNA-synthesizing and dividing cells in the lymphoid organs of the rat. Ann. Nutr. Aliment. 32:15-40, 1978.

15. STIEHM, E.R. Humoral immunity in malnutrition. Fed. Proc. 39:3093-3097, 1980.

16. WRONSKI, A. and VON DER DECKEN, A. Protein synthesis with special reference to S-100 protein in brain slices from rats receiving a restricted protein supply. Acta Physiol. Scand. 97:20-30, 1976.

17. KUBÁT, B., MORÉN, G.M. and VON DER DECKEN, A. DNA-dependent RNA polymerases from rat brain, and RNA synthesis in glial and neuronal cell-enriched fractions, after protein restriction. J. Neurochem. 31: 1143-1148, 1978.

18. FERNANDES, G., YUNIS, E.J. and GOOD, R.A. Influence of diet on survival of mice. Proc. Natl. Acad. Sci. USA 73:1279-1283, 1976.

19. GOOD, R.A., WEST, A. and FERNANDES, G. Nutritional modulation of immune responses. Fed. Proc. 39:3098-3104, 1980.

20. KRAMER, T.R. and GOOD, R.A. Increased in vitro cell-mediated immunity in protein-malnourished guinea pigs. Clin. Immunol. Immunopathol. 11:212-228, 1978.

21. ERICKSON, K.L., GERSHWIN, M.E., McNEILL, C.J., OSSMANN, J.B. and CANOLTY, N.L. Effects of low dietary protein concentration and energy deprivation on lymphocyte transformation in melanoma-bearing mice. J. Nutr. 109:1893-1900, 1980.

22. DUTTON, R.W. T lymphocyte subsets and interactions. Fed. Proc. 39:3109, 1980.

23. SWAIN, S.L. Association of Ly phenotypes, T cell function and MHC recognition. Fed. Proc. 39:3110-3113, 1980.

NUTRITION RESEARCH, Vol. 1, pp. 277-287, 1981
0271-5317/81/030277-11$02.00/0 Printed in the USA.
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THE EFFECT OF DIETARY FAT AND SALT ON BLOOD PRESSURE, RENAL AND AORTIC PROSTAGLANDINS[1]

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ABSTRACT

The pressor effects of fat and salt were examined in male Sprague-Dawley rats. Rats fed high carbohydrate (5% corn oil), high salt (8% NaCl) diets demonstrated significant elevations in blood pressure within one week of diet introduction which persisted throughout the 9 week experimental period. High saturated fat (5% corn oil-15% coconut oil) diets promoted a significant elevation in blood pressure, irrespective of the level of dietary salt. High dietary salt, as opposed to basal levels, tended to decrease the blood pressure of rats fed a diet containing all unsaturated fat (20% corn oil). The greatest percent increase in the renal vasodilator prostaglandin E_2 was measured in rats fed the high unsaturated-basal salt diet compared to all other dietary treatments. A hypervolemic effect of high levels of dietary salt was demonstrated in the high carbohydrate, all unsaturated fat and high saturated fat groups. These results demonstrate that the amount and type of dietary fat interact with the level of dietary salt to influence blood pressure in rats.

Key Words: Hypertension, Fat, Salt, Prostaglandins

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INTRODUCTION

Numerous clinical (1,2), epidemiological (3,4) and experimental (5) studies implicate high salt intake in the development of essential hypertension. Recent evidence suggests that the hypertensive effect of high levels of dietary salt may be modified by varying the level of dietary fat (6-10). In addition, we have reported that diets high in saturated fat elicit hypertensive effects similar to diets high in salt (9,10). However, the relative importance of the type of dietary lipid in causing increased blood pressure has only recently become evident (10).

It has been suggested that different types of dietary fat may alter prostaglandin metabolism and subsequently may affect blood pressure (11,12). Rats fed high levels of saturated fat excrete decreased levels of urinary prostaglandins (7). Prostaglandins have been identified as potent vasoactive substances: prostaglandin E_2 is classified as a vasodilator and prostaglandin $F_{2\alpha}$ is considered a vasoconstrictor (13). The purpose of this investigation was to examine how the amount and type of dietary fat interacts with the level of dietary salt to influence blood pressure. The effect of dietary treatment on the levels of the renal and aortic prostaglandins E_2 and $F_{2\alpha}$ was also determined.

MATERIALS AND METHODS

Animals and Diets

Male ARS Sprague-Dawley rats (Madison, WI), approximately 6 wks. of age, were fed a high carbohydrate (HCARB; 5% corn oil) basal salt (BS; 0.15% NaCl) diet for a period of one week before subgroups were randomly selected and exposed to one of six dietary regimens for a period of nine weeks duration. The dietary regimens are designated AUSAT (all unsaturated fat 20%; corn oil), HSAT (high saturated fat; 5% corn oil-15% coconut oil), HCARB (high carbohydrate; 5% corn oil), with either HS (high salt; 8.0% NaCl) or BS (basal salt; 0.15% NaCl). A detailed description of the diets is listed in Table 1.

Food and water were available ad libitum. Water and food consumption, and weight gain were recorded on a weekly basis. We have previously reported that under these experimental conditions rats consume a similar quantity of caloric as well as non-caloric constituents, regardless of the caloric density of the diet (9,10). Blood pressure was determined indirectly using a tail cuff electro-sphygmomanometer, as previously described (9,10).

Prostaglandin Analyses

All rats were killed by decapitation after nine weeks of exposure to one of the various dietary treatments. Kidneys and aortas were quickly excised, frozen in a dry ice-acetone bath and stored on dry ice for no longer than three days (14). Before analysis, tissues were weighed and homogenized in 0.1M phosphate buffered saline (pH 7.4). Indomethacin (0.5mg) was added to each homogenized sample (14). Aliquots (2ml) of kidney and aorta were used for analysis of prostaglandin E_2 (PGE_2) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) by the method of Behrman et al. (14). All samples were assayed in duplicate.

TABLE 1
Diet Composition

Constituent	Amount (g/100 g of diet) HCARB BS	HCARB HS	AUNSAT BS	AUNSAT HS	HSAT BS	HSAT HS
Casein	20.0	18.2	24.0	22.0	24.0	22.0
Sucrose	48.2	45.2	19.8	18.0	19.8	18.0
Fat: Corn	5.0	4.6	24.0	22.0	6.0	5.5
Coconut	0	0	0	0	19.0	16.5
Choline Chloride	0.20	0.18	0.25	0.22	0.25	0.22
Salt Mix[1]	4.1	3.6	4.8	4.4	4.8	4.4
Vitamins[2]	2.49	2.46	2.60	2.55	2.60	2.55
Corn Starch	10.0	9.2	12.0	11.0	12.0	11.0
Cellulose	10.0	9.2	12.0	11.0	12.0	11.0
D,L-Methionine	0.29	0.28	0.36	0.33	0.36	0.33
NaCl	0.15	7.3	0.18	8.8	0.18	8.8
Cal/gm	3.65	3.39	4.47	4.09	4.47	4.09

[1]The salt mix contained (in %) - sodium chloride, 13.9325; potassium phosphate 38.8967; magnesium sulfate, 229.2; calcium carbonate, 38.1442; ferrous sulfate, 2.696; potassium iodide, 0.079; magnesium sulfate, 0.4453; zinc chloride, 0.0259; copper sulfate, 0.0475; cobalt chloride, 0.0022.
[2]The vitamin mix contained (per kilo of diet) - Vitamin A concentrate, 19822 IU; Vitamin D concentrate, 2200 IU; alpha tocopherol, 110 mg; ascorbic acid, 991 mg; inositol, 110 mg; choline chloride, 1.65g; menadione, 49.5 mg; p-aminobenzoic acid, 110mg; niacin, 99.0 mg; riboflavin, 22 mg; pyridoxine hydrochloride, 22 mg; thiamine hydrochloride, 22 mg; calcium pantothenate, 66 mg; biotin, .44 mg; folic acid, 1.98 mg; vitamin B_{12}, 0.03 mg.

Statistical Analyses

Experimental results were analyzed using a two-way analysis of variance. Significance of differences among individual points was determined using Ducan's Multiple Range test at the 95% confidence interval (15).

RESULTS

Table 2 shows the effect of various diets on weekly salt consumption. All high-salt groups consumed a similar amount of salt per week except at week 1 when the AUNSAT-HS group consumed significantly more salt than did the HCARB-HS and HSAT-HS groups. Also, there were no significant differences in the amount of salt consumed among the basal salt groups (data not shown). Overall, there were no significant differences in body weight gain or in caloric consumption among the dietary groups.

Blood Pressure

The effect of different levels of salt intake on blood pressure is shown in Fig. 1a. Rats fed the HCARB-HS diet demonstrated a significant elevation in blood pressure within one week of diet introduction. This increase remained significant throughout the

TABLE 2
The Effect of Various Diets on Weekly Salt Consumption

Time (weeks)	NaCl Consumed (g/rat/wk)[1] HCARB-HS	AUNSAT-HS	HSAT-HS
1	12.6±0.7	14.8±1.7	10.6±1.0
2	14.5±1.0	14.9±2.7	13.6±1.0
3	16.6±0.8	16.5±2.7	16.2±1.0
4	18.7±0.7	17.9±3.3	18.4±2.2
5	17.5±0.8	15.5±3.3	17.0±1.2
6	18.9±1.0	18.0±4.3	20.5±3.4
7	21.3±2.2	17.6±3.9	19.6±4.6
9	21.9±3.1	17.8±3.1	17.5±0.9

[1]Each value represents the mean ± S.E.M. for 7 rats. No significant differences among groups were observed except at week 1 where AUNSAT-HS was significantly ($p \leq 0.05$) greater than either of the other two groups.

entire experimental period. Rats comsuming the AUNSAT-HS diet exhibited blood pressures that tended to be lower than observed in the AUNSAT-BS group, although significance was inconsistently achieved. Except at week 5, the blood pressure in the HSAT-BS and HSAT-HS groups were similar.

The effect of different dietary fats on blood pressure is demonstrated in Fig. 1b. Rats consuming the HCARB-BS diet demonstrated significantly lower blood pressures than rats fed either the AUNSAT-BS or the HSAT-BS diet through week 6 of dietary treatment. By week 7, blood pressure in the HSAT-BS group was significantly higher than in the AUNSAT-BS and HCARB-BS groups. By week 9, blood pressure among all BS groups was significantly different: the HSAT-BS group exhibited the highest blood pressure, the HCARB-BS the lowest, and the AUNSAT-BS group was intermediate. The HCARB-BS and AUNSAT-HS groups demonstrated similar blood pressures throughout the 9-week experimental period. After week 2, rats consuming the HSAT-HS diet tended to have significantly higher blood pressures than either the AUNSAT-HS or the HCARB-HS group.

The weekly water consumption and blood pressure appeared to covary in rats exposed to the HCARB-HS, AUNSAT-HS and HSAT-HS diets (Fig. 2). It is interesting that rats fed the AUNSAT-HS and HSAT-HS diets tended to consume more water than the rats in the HCARB-HS group.

Prostaglandin Analyses

Table 3 shows the percent change of renal PGE_2 and $PGF_{2\alpha}$ levels in rats consuming the various dietary regimens. Rats consuming the AUNSAT-BS diet demonstrated the greatest percent increase (90.6%) in renal PGE_2 levels as well as the greatest percent increases in $PGE_2/PGF_{2\alpha}$ levels. The role of diet in altering renal $PGF_{2\alpha}$ levels was minimal in relation to changes in renal PGE_2 levels in all rats examined. All groups except the HSAT-BS group had increases in renal $PGE_2/PGF_{2\alpha}$ levels when compared to that observed in the HCARB-BS group.

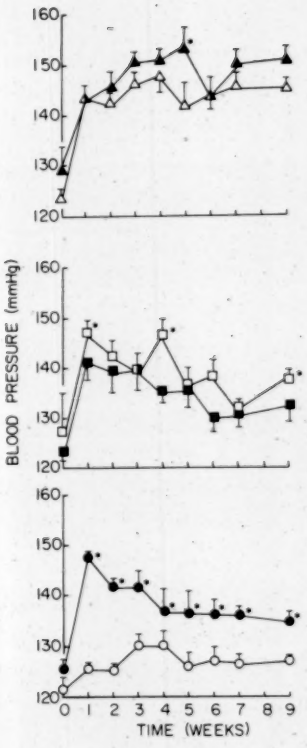

FIG. 1a
The effect of dietary salt on blood pressure when fed in conjunction with different fats. Each point represents the mean systolic blood pressure of 7 rats + S.E.M. Significant ($p \leq 0.05$) differences between the HS and BS groups are designated by an asterisk. Comparisons are made within each time period.

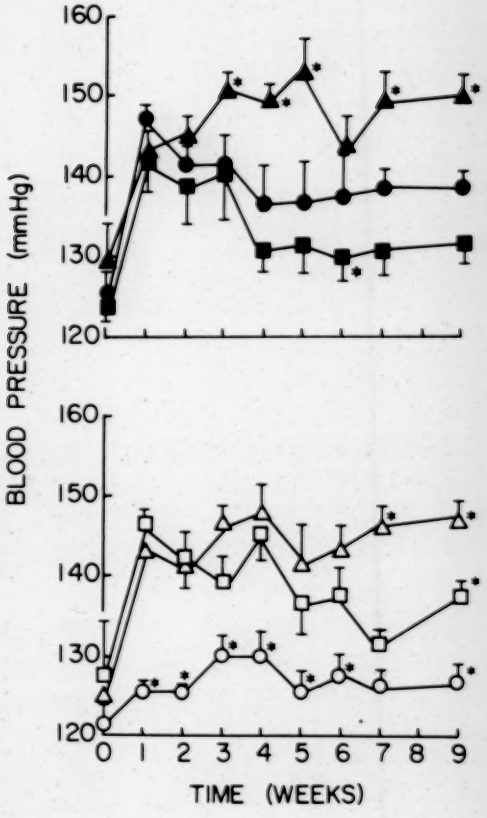

FIG. 1b
A comparison of effects of various fats on systolic blood pressure. The data are taken from Fig. 1a. An asterisk indicates that a point is significantly different from the other two dietary groups within the respective time period.
-○-HCARB-BS; -●-HCARB-HS;
-□-AUNSAT-BS; -■-AUNSAT-HS;
-△-HSAT-BS; -▲-HSAT-HS

The influence of the experimental diets on aortic PGE_2 and $PGF_{2\alpha}$ levels is also expressed in Table 3. Large percent increases in aortic PGE_2 levels were observed in all dietary groups when compared to that of the controls (HCARB-BS). To a lesser extent, aortic $PGF_{2\alpha}$ levels also increased in all dietary groups, except the AUNSAT-BS group, in comparison to that of the controls. All dietary treatment groups demonstrated increases in aortic $PGE_2/PGF_{2\alpha}$ levels when compared to the HCARB-BS group.

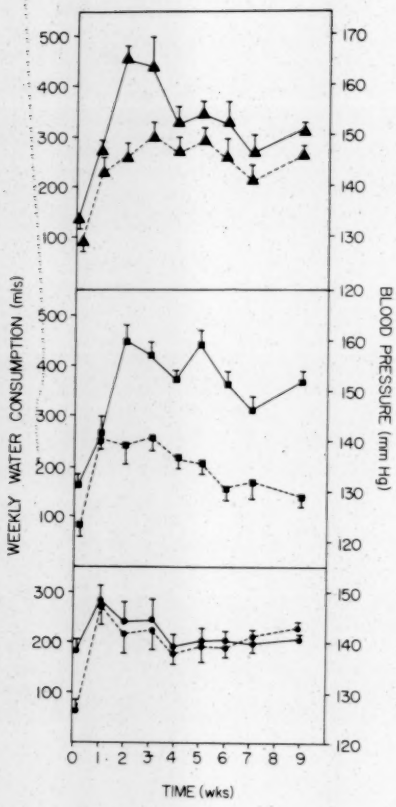

FIG. 2
The relationship between blood pressure and water consumption of rates exposed to the HS diets. The blood pressure values are taken from FIG. 1. The symbols denote the same dietary treatments as in the previous figure and each point represents the mean ± S.E.M. for 7 rats. Water consumption is represented by the solid line and blood pressure by the dashed line.

DISCUSSION

Results from this experiment suggest that the blood pressure regulating effects of fat are dependent on the type as well as the level of fat in the diet. High saturated fat diets, irrespective of the level of dietary salt, induced sharp increases in blood pressure (20%) which persisted throughout the 9-week experimental period. Although a 15% increase in blood pressure was

Table 3
The Influence of Various Diets on Renal and Aortic Levels of PGE_2 and $PGF_{2\alpha}$[1]

	% Change[2]					
	HCARB		AUNSAT		HSAT	
Prostaglandin	BS	HS	BS	HS	BS	HS
Kidney						
E_2	53±8[3]	+34	+91	-4	-25	+23
F_2	28±8	+12	+ 4	-4	+ 5	- 7
$E_2/F_{2\alpha}$	2±0.3	+11	+76	+2	-26	+27
Aorta						
E_2	145±4	+141	+426	+388	+760	+1275
F_2	140±80	+107	- 6	+ 92	+ 24	+ 499
$E_2/F_{2\alpha}$	1.5±0.8	+ 73	+360	+ 73	+133	+ 67

[1] Kidneys from 2-3 rats were pooled for analysis yielding 3 samples per dietary group. Aortas from 3-4 rats were pooled for analysis yielding 2 samples per dietary group.
[2] Percent change from the HCARB-BS group was calculated using the mean values.
[3] Values are listed as ng/gm tissue and represent the mean ± S.E.M.

observed within one week of introducing the AUNSAT diets, the hypertensigenic effects diminished over time with only an 8% elevation in blood pressure remaining after 9 weeks. It is interesting to note that while the HSAT-HS group tended to have slightly elevated blood pressures relative to the HSAT-BS group, the reverse was observed in the AUNSAT groups; i.e. the AUNSAT-HS group tended to have lower blood pressure compared to the rats fed the AUNSAT-BS diet. At present, no explanation for this observation is apparent.

The results reported herein extend earlier observations that fat and salt influence blood pressure (6-10). A major difficulty frequently encountered with previously reported studies is that the interaction between fat and salt was not examined quantitatively. Salt was presented in the drinking water without reporting salt solution consumption. Consequently, the amount of sodium chloride consumed by the rats can not be calculated. We observed that rats consuming the high salt diets, irrespective of the level and type of dietary fat, consumed similar quantities of salt per week (Table 2). Therefore, the observed changes in blood pressure in the dietary groups can not be entirely accounted for by the level of salt consumed.

Previous investigators have demonstrated a blood pressure regulating effect for dietary lipids. Gardey et al. (16) observed significant elevations in the blood pressure of rabbits fed 20% coconut oil diets. In addition, New Zealand white rabbits fed diets containing palm oil demonstrated a greater increase in blood pressure than rabbits fed safflower oil diets (17). Palm oil contains a greater percentage of saturated fatty acids than safflower (18). It has also been reported that albino rats fed an essential fatty acid deficient diet have higher blood pressures than rats fed an essential fatty acid sufficient diet (19). When essential fatty acid deficient rats were fed elevated levels of unsaturated fatty acids, their blood pressures dropped to levels similar to those measured in the essential fatty acid sufficient group (19). In our experiments, the elevated blood pressures observed in the HSAT groups can not be attributed to a fatty acid deficiency because the HSAT diets contained 5% corn oil (Table 1).

Ten Hoor and Van de Graff (7) reported an 8% increase in blood pressure when Wistar rats were fed a high coconut oil, high salt diet. We observed a 20% increase in blood pressure in the HSAT-HS group. This discrepency may be attributed to several factors. Ten Hoor and Van de Graff presented salt in the drinking water (7) whereas we presented the salt as a solid mixed into the diet. In addition, we exposed the rats to dietary treatment at an earlier age than did Ten Hoor and Van de Graff and it has been reported that weanling rats are more susceptible to the hypertensive effects of dietary sodium chloride relative to older animals (20). Most importantly, the Wistar strain of rat, unlike the Sprague-Dawley strain, is reported to be resistant to salt induced changes in blood pressure (21).

It has been suggested that the pressor effects of various types of lipids may be mediated through changes in prostaglandin metabolism (7,8,22). It appears that prostaglandins influence blood pressure in at least two ways: by regulation of renal sodium ex-

cretion either directly or by alterations in renin and aldosterone secretion, and by direct action on blood vessels, i.e. through vasodilation or vasoconstriction (22-24).

The data reported here (Table 3) demonstrate that rats fed the AUNSAT-BS diet exhibit the greatest percent increase in renal PGE_2 levels and renal $PGE_2/PGF_{2\alpha}$ ratios when compared to the HCARB-BS group. $PGF_{2\alpha}$ levels in the AUNSAT-BS group remained relatively constant. Our results support those of Hoffman et al. (2) who reported that PGE_2 efflux from isolated perfused kidneys was higher in linoleic acid-fed rats than in rats fed a linoleic acid-free diet. These investigators also reported that $PGF_{2\alpha}$ efflux remained unchanged (25) and agrees with our findings of relatively constant levels of renal $PGF_{2\alpha}$.

Elevated renal PGE levels have been shown to cause an increased release of renin and angiotensin (23). Renin and angiotensin have been identified as hypertensive agents by numerous investigators (23,26,27). However, the hypertensive effect of elevated renin and angiotensin levels may be ameliorated by the peripheral vasodilationary effect of elevated levels of PGE_2 or other vasoactive prostaglandins such as PGI_2 (28) in the AUNSAT-BS group. This may account for the different blood pressures observed in the AUNSAT-BS and the HSAT-BS groups. The greatest percent decrease in renal PGE_2 and $PGE_2/PGF_{2\alpha}$ levels was observed in the HSAT-BS group.

Although prostaglandins have been shown to be potent vasoactive substances, the exact mechanism by which prostaglandins modulate blood pressure has not been clearly delineated. Comberg et al. (9) suggested that blood pressure is controlled by several factors including the rate of prostaglandin synthesis, plasma renin levels, and the amount of fat and salt ingested. It is possible that the role of renal prostaglandins becomes diminished in relation to other regulatory mechanisms controlling extracellular fluid volume in the high fat, high salt groups. All HS groups drank significantly more water than their respective BS control groups. In addition, weekly changes in blood pressure in all HS groups directly covaried with weekly water consumption (Fig. 3). These results suggest that the hypervolemic effect of high levels of dietary salt may play a compensatory role in regulating blood pressure in the high salt groups.

Previous studies have identified the role of dietary salt in the development of hypertension (2,3,5). These experiments utilized high carbohydrate diets and varied the level of salt in the diet or in the drinking water. Results reported here confirm the concept that high levels of dietary salt play a key role in affecting blood pressure if fed in conjunction with a high carbohydrate diet. Rats fed the HCARB-HS diet demonstrated significantly elevated blood pressures throughout the 9-week experimental period (Fig. 2). However, our results extend and confirm earlier observations that the blood pressure regulating effect of salt is minimized if fed in conjunction with high levels of unsaturated fat. Furthermore, feeding a diet that contains high levels (20%) of saturated fat appears to be more detrimental in causing increases in blood pressure than a diet that contains 8% NaCl. Our

data also lend support to the suggestion that renal prostaglandins, in conjunction with other mechanisms that regulate blood volume, may play a role in mediating dietary influences on blood pressure. The relationship between prostaglandins and hypertension warrants further investigation and dietary models promise to be useful for ellucidating the interactions among blood pressure, lipid, NaCl, and prostaglandins.

ACKNOWLEDGMENTS

The authors wish to thank Dr. H. Behrman of Yale University Medical School for the use of his laboratory for the prostaglandin analyses and Dr. M. Kare of the Monell Chemical Senses Center for his continued support. A portion of this work was supported by USDA Grant No. 59-32U4-0-3.

REFERENCES

1. MORGAN, T., GILLIES, A., MORGAN, G., ADAM, W., WILSON M. and CARNEY, S. Hypertension treated by salt restriction. Lancet, 4: 227-233, 1978.

2. SWAYE, P.S., GIFFORD, R.W. and BERRETTONI, J.N. Dietary salt and essential hypertension. Amer. J. Cardiol., 29: 33-38, 1972.

3. DAHL, L.K. Salt and hypertension. Amer. J. Clin. Nutr., 25: 231-244, 1972.

4. PRIOR, I.A.M., EVANS, J.G., HARVEY, H.P.B., DAVIDSON, F. and LINDSEY, M. Sodium intake and blood pressure in two Polynesian populations. New Eng. J. Med., 279: 515-520, 1968.

5. MENEELY, G.R. and BALL, C.O.T. Experimental epidemiology of chronic sodium chloride toxicity and the protective effect of potassium chloride. Amer. J. Med., 25: 713-725, 1958.

6. TRIEBE, G., BLOCK, H.U. and FORSTER, W. On the blood pressure response of salt loaded rats under different content of linoleic acid in food. Acta. Biol. Med. Germ., 35: 1223-1224, 1976.

7. TEN HOOR, F. and VAN DE GRAAF, H.M. The influence of a linoleic acid-rich diet and acetyl salicylic acid on NaCl induced hypertension, Na^+- and H_2O-balance and urinary prostaglandin excretion in rats. Acta. Biol. Med. Germ., 37: 875-877, 1978.

8. VERGROESEN, A.J., FLEISCHMAN, A.I., COMBERG, H.U., HEYDEN, S. and HAMES, C.G. The influence of dietary linoleate on essential hypertension in man. Acta. Biol. Med. Germ., 37: 879-883, 1978.

9. SMITH-BARBARO, P., QUINN, M.R., FISHER, H. and HEGSTED, D.M. Pressor effects of fat and salt in rats. Proc. Soc. Exp. Biol. Med. 165: 283-290, 1980.

10. SMITH-BARBARO, P., FISHER, H., QUINN, M.R. and HEGSTED, D.M. The effect of varying polyunsaturated to saturated fat ratios on salt-induced hypertension in rats. Nutr. Reports Intl. 22: 759-770, 1980.

11. COMBERG, H.U., HEYDEN, S., HAMES, C.G., VERGROESEN, A.J. and FLEISCHMAN, A.K. Hypotensive effect of dietary prostaglandin precursor in hypertensive man. Prostaglandins, 15: 193-197, 1978.

12. LEARY, W.P., LEDINGHAM, J.G. and VANE, J.R. Impaired prostaglandin release from the kidneys of salt-loaded and hypertensive rats. Prostaglandins, 7: 425-432, 1974.

13. WEBER, P.C., LARSSON, C. and SCHERER, B. Prostaglandin $E_2$9-ketoreductase as a mediator of salt intake-related prostaglandin-renin interaction. Nature, 266: 65-66, 1977.

14. JAFFEE, B.M. and BEHRMAN, H.R. In: Methods of Hormone Radioimmunoassay. Academic Press, Inc., N.Y., 1979, pp. 20-23.

15. MEDENHALL, W. Introduction to the probability of statistics. 3rd edition. Duxbury Press, Belmont, California, 1971, pp. 331-356.

16. GARDEY, T., BURSTYN, P.G. and TAYLOR, T.G. Fat induced hypertension in rabbits. 1. The effects of fibre on the blood pressure increase induced by coconut oil. Proc. Nutr. Soc., 37: 97A, 1978.

17. KENNEDY, M., BURSTYN, P.G. and HUSBARDS, D.R. Fat induced hypertension in rabbits. 2. The effect of feeding diets containing high concentrations of safflower oil and palm oil. Proc. Nutr. Soc., 31: 1, 1978.

18. ALTMAN, P.L. and DITTMER, D.S. In: Biology Data Book. Fed. of the Amer. Soc. Exp. Biol., Bethesda, Maryland, 1964, pp. 380-381.

19. ROSENTHAL, J., SIMONE, P.G. and SILBERGLEIT, A. Effects of prostaglandin deficiency on natriuresis, diuresis and blood pressure. Prostaglandins, 5: 435-440, 1974.

20. WILSON, R.B., SMITH, D.M. and NEWBERNE, P.M. Excess sodium chloride intake in neonatal rats. Archives of Path., 96: 372-376, 1973.

21. TORII, K. In: Biological and Behavioral Aspects of Salt Intake (Kare, M.R., Fregly, M.J. and Bernard, R.A., eds.) Academic Press, Inc., 1980, pp. 345-366.

22. BRICKER, N.S., KLAHR, S., PURKERSON, M., SCHULTZE, R.G., AVIDI, L.V. and BIRGE, S.J. In vitro assay for a humoral substance present during volume expansion and uraemia. Nature, 219: 1058-1059, 1969.

23. LEE, J.B. Cardiovascular-renal effects of prostaglandins. Arch. Inter. Med., 133: 56-76, 1974.

24. LEE, J.B., PATAK, R.V. and MOOKERJEE, B.K. Renal prostaglandins and the regulation of blood pressure and sodium and water homeostasis. Amer. J. Med., 60: 798-816, 1976.

25. HOFFMANN, P., TAUBE, C., PONICKE, K., FORSTER, W., SAMOVA, L., ORBETZOVA, V., and DAVIDOVA, F. Influence of linoleic acid content of the diet on arterial pressure of salt loaded rats. Acta. Biol. Med. Germ., 37: 863-867, 1978.

26. MARX, J.L. Hypertension: A complex disease with complex causes. Science, 194: 821-825, 1976.

27. GUYTON, A.C., COLEMAN, T.G., COWLEY, A.W., SCHEEL, K.W., MANNING, R.D. and NORMAN, R.A. Arterial pressure regulation. Amer. J. Med., 52: 584-594, 1972.

28. WEEKS, J.R. and COMPTON, L.D. The cardiovascular pharmacology of prostaglandin (PGI_2) in the rat. Prostaglandins, 17: 501-513, 1979.

NUTRITION RESEARCH, Vol. 1, pp. 289-301, 1981
0271-5317/81/030289-13$02.00/0 Printed in the USA.
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OBESITY IN INFANTS, CHILDREN, AND ADOLESCENTS IN THE UNITED STATES III. THERAPY AND PREVENTION: INDIVIDUAL, FAMILY, AND COMMUNITY

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ABSTRACT

The management of obesity in children and adolescents has not received intensive investigation. Attempts at modifying behavior have had only limited success; published results have been biased by voluntary participation in controlled studies, by use of highly restrictive diets, and by the study of children with lesser degrees of obesity. A major difficulty in the evaluation of more radical therapies, such as surgery or highly restrictive diets, is that the risks of therapy must be balanced against the risks of continuing disease. Information on both is sparse. Review of the epidemiologic variables associated with obesity suggests that family factors have a significant effect on both the onset and course of the disease. Family therapy therefore deserves more critical evaluation.

KEY WORDS: Obesity, therapy, prevention, behavioral modification, surgery

The existing limited understanding of the causality and natural history of obesity in children and adolescents restricts our ability to design rational interventions, or to appraise results of therapy or prevention objectively. More specifically, programs aimed at preventing obesity are not likely to be effective until directed at demonstrated causes of origin. Likewise, the most acceptable evidence of successful therapy is a frequency of remission significantly greater than that observed in a control population.

TREATMENT OF THE INDIVIDUAL AND FAMILY

Therapeutic methods currently used to manage obesity in children and adolescents are presented in Table 1. Aside from surgical intervention and drug therapy, most are time-consuming and difficult to quantify. In addition, they follow no common model of medical therapy, which may explain in part the few available clinical reports of management of obesity in children and adolescents.

Drug Therapy

Drug therapy has little short term value, does not promote long term remissions, and compliance is poor (1). Furthermore, some drugs retard growth (2); the risk of abusehas prompted the Committee on Nutrition of the American Academy of Pediatrics to discourage their use (3). At the present time, drug therapy has no role in the treatment of obesity in children or adolescents.

Table 1

Treatment Regimens for Obesity in Children and Adolescents.

A. Drug Therapy
B. Education
C. Dietary Therapy
D. Behavioral Modification
   1. Of individual behavior
   2. Of family behavior
E. Family Therapy
F. Surgery

Education

With the exception of therapy directed at altering patient behavior, studies applying and evaluating a single therapeutic modality are rare. Most programs combine multiple treatments, such as education and diet; education alone has not been compared with other regimens. Counseling for problems other than obesity reportedly produced lower response rates and poorer weight maintenance in adolescents, compared to counseling and dietary therapy (4). The same study indicated that treatment of adolescents in groups had no advantage over individualized management. However, the procedures used for group or individual therapy were not described.

Dietary Therapy

Dietary therapy is necessarily the cornerstone of weight reduction therapy. Far greater caloric deficits can be achieved by restricting caloric intake than by increasing energy expenditure. Using a balanced calorie deficit diet in which caloric intake is reduced to two-thirds of the usual daily intake, we have observed no adverse effects on growth or other biochemical parameters in a large number of children. Somewhat surprisingly, published experience with dietary therapy in children and adolescents is small.

The only study reporting individual responses to dietary therapy of unselected patients (5) found that 60% of treated adolescents gained weight, 10% lost between 2.5 and 4.9 kg, and 20% lost over 5 kg. However, detailed descriptions of therapy, the duration of treatment, and the percentage of adolescents who maintained lost weight were not presented. As expected, more restrictive diets produced greater weight losses, but success in maintaining long term weight loss was still low (6).

At least one other study compared the results of several different treatment modalities (7), but reported weight losses as average values, making impossible a comparison of degree and frequency of success. Twenty-seven boys in excess of 115% of ideal body weight were treated with similar diets consisting of: a) 4800 kilocalories without snacks; b) 4800 kilocalories divided in six meals; c) exercise and 4800 kilocalories divided in six meals; or, d) behavioral modification of speed of eating and 4800 kilocalories divided in six meals. The first two groups did not differ in amounts of weight lost, but appreciably greater losses ensued with exercise, and still more with added behavior modification. The results are intriguing, but the limited information precludes definitive conclusions.

Dietary management deserves more intensive investigation. Most reports neither specify the methods of patient selection nor do they include individual responses to therapy. Moreover, both short term and long term success rates are low. Growth retardation is an important side effect of highly restrictive dietary management (8, 9); consequently, such diets should be employed cautiously.

In contrast to adults (10), short term adaptations of nitrogen balance did not occur in four of twelve children receiving low calorie, carbohydrate-free diets (11). One of these four children developed lymphopenia; retinol binding protein, C_3, and transferrin levels fell in the three patients tested (12). Furthermore, carbohydrate may be more beneficial than fat in promoting nitrogen balance among children and adolescents (12).

Since weight reduction for the severely obese is a prolonged process, and since rapid weight loss may improve compliance, a restrictive diet which also enhances protein metabolism appears essential. Nonetheless, any therapy with accompanying hazards must be judged by the risks of continuing disease. Currently, neither the risks of obesity nor the hazards of therapy have been well quantified. Until these risks are determined with greater precision, highly restrictive diets in children and adolescents must be used with caution.

Behavioral Modification

In recent years, attention has focused on behavioral modification as a mode of treatment for weight reduction of obese children and adolescents (13). Although the familial and cultural practices promoting obesity have yet to be explicitly identified, nevertheless, these factors have a recognized ability to maintain or accentuate obesity. Behavioral management is a practical and logical approach.

Some behavioral modifications designed to achieve weight loss are presented in Table 2. Self-monitoring requires that the patients become aware of what, where, when, and why they eat as they do (14-16). Stimulus control refers to the recognition and control of all aspects of the eating environment (15, 17). Changes in behavior deserve prompt positive reinforcement, both by parental recognition and encouragement as well as by mutually acceptable rewards (15). Cognitive restructuring refers to altering the internal monologues that play a role in the maintenance and control of obesity (18) by substituting positive statements for negative thoughts about weight, approaches to losing weight, excuses, goals, and attitudes toward food (13, 17, 18).

Table 2

Components of Behavioral Modification in the Management of Obesity of Children and Adolescents by Behavior Modification.*

A. Self Monitoring
B. Stimulus Control
C. Slowing the Act of Eating
D. Reinforcement
E. Cognitive Restructuring

*Adapted from Brownell and Stunkard (13).

Several available outpatient studies that illustrate the generally limited success of current therapy are summarized in Table 3. Since published data from unselected patients are few, the studies considered are probably biased toward more favorable results. Treatment programs varied greatly. The proportion of children who lost 5 kg or more was deceptively large since many treatments either employed highly restrictive diets (16), included preselected patients, or involved the less obese (15). The single report of dietary management alone (5) better represents the expected results of general medical therapy of unselected patients; only 20% of the children and adolescents lost more than

5 kg. Long term remissions, defined as the proportion who maintained substantial weight losses or continued to lose weight, approximated 20%.

Family Therapy

In 1978, we developed a program at the Boston Children's Hospital Medical Center that incorporated most of the published behavioral techniques effective in inducing weight loss in obese children. By comparison to the other results listed in Table 3, our results were discouraging. Nonetheless, our results were comparable to those found in the only other study of unselected patients (5), or in children of the same age and degree of obesity (14).

Table 3

Results of weight Reduction Programs for Children and Adolescents.

Author and Reference	N*	% IBW †	Method	Percentage Patients Losing > 5 kg
Aragona et al, 1975 (15)	7	114%	Behavioral Modification	72%
Alley et al, 1968 (5)	30	145%	1,000 kcal diet	20%
Rivinus et al, 1976, (14)	9	172%	Parent and child group meetings	22%
Heyden et al, 1973 (16)	27	N.D.‡	700 kcal diet, with intermittent fasting, group counseling	44%
	8	N.D.	700 kcal diet, with intermittent fasting, individual counseling	88%
Gross et al, 1976 (17)	10	139%	Behavioral self control	30%
Dietz, unpublished data, 1978	71	164%	Multiple	11%

* Number studied
† Percentage ideal body weight for height and sex
‡ Not done

Our low success rate, despite the most optimal therapy available, suggested that there existed a subgroup of obese children likely to respond to therapy, regardless of the management employed. Furthermore, by determining the characteristics of the non-responders , we felt that we might be able to revise our program to develop more effective therapy for this group.

The initial problem we encountered in our analysis was a "drop out" rate of over 50%. This rate of default was comparable to the range of 24% to 78% observed in other studies (19-23). To normalize our data, we therefore compared rates of weight reduction. As shown in Table 4, rates of weight loss were significantly lower among children with one or both parents obese, and among children in single parent families. Furthermore the effects of parental obesity and marital status on rates of weight loss were independent.

Table 4

Effect of Parental Obesity and Marital Status on Mean Rates of Weight Loss of Children Attending a Pediatric Weight Control Program.

Groups Compared	Number of Patients	Mean Weight Loss Per Session (Kg + S.D.)
I. Parental Obesity		
Neither parent obese	14	-.55 ± .45*
One parent obese	27	-.26 ± .57
Both parents obese	26	-.22 ± .47
II. Marital Status		
Parents married	37	-.39 ± .46‡
Parents divorced or separated	25	-.06 ± .60

* Significantly greater rate of weight loss than children with one or both parents both parentsobese (ANOVA, $F(1,67) = 7.1$, $< .01$). Four children excluded from analysis because of parental death.

‡ Significantly greater rate of weight loss than children whose parents are divorced or separated ($t = 2.37$, $p < .01$, one tailed test). Nine children excluded from analysis because of parental death or unclear marital status.

The demonstration that family variables affect the course of the disease, and the epidemiologic evidence that family factors are also associated with the onset of the disease, emphasizes the need for further investigation of behaviors within families of obese children and adolescents.

Relevant family functions that promote weight loss may be grouped into the interrelated categories of behaviors related to food, and interactions between family members. Family practices related to eating are shown in Table 5. Modification of maladaptive food habits, in conjunction with hypocaloric dietary therapy promises weight loss. Weight loss indicates that the family is capable of changing to support the needs of the affected member. Failure signals the need for further investigation, and, often, additional necessary interventions within the family.

In collaboration with Dr. Barry Dym, of the Family Institute of Cambridge, we have observed that a child's obesity often serves a homeostatic function within families. Attempts to induce weight reduction without altering the family's pattern of interactions are rarely successful. For this reason, psychotherapy to treat a child's obesity may have adverse consequences by perpetuating the notion that the affected child is responsible for the disease.

Family therapists in our clinic have begun to explore in greater detail the features of families with obese children. A reversed heirarchy appears characteristic of many such families, particularly those of younger children. Parents are often controlled by their child, and have difficulty limiting the obese child's food intake. When the hierarchy has been changed, either by restoring parental control, or by the fortuitous placement of a child in a family with an intact hierarchy, prompt weight reduction has ensued.

Table 5

Family Practices that Enhance Obesity or Prevent Weight Reduction

I. Food Related Behaviors
   A. Food Purchasing
   B. Food Sources
   C. Food Preparation
   D. Food Consumption
      1. Serving
      2. Rates of eating
      3. Plate size
      4. Distraction

II. Family Interactions

A frequent finding in families with older children has been overinvolvement of the parent with the obese child or adolescent. Increasing the autonomy of the adolescent often induces weight loss, but additional work on motivation may be required. Although our experience with this approach is limited, our results to date suggest that this family approach will increase compliance with both recommendations for dietary therapy and implementation of techniques to alter behavior.

Surgery

Surgery for obesity in children and adolescents is justified when severe effects of the disease are present, and after more conservative management has failed. Jejunoileal bypass is accompanied by significant aftereffects (24); only gastric bypass surgery should be recommended (25). Nonetheless, experience with gastric bypass surgery in adolescents is limited to one report (26). Postoperative mortality in 1585 adult patients was 1.6% (27), but complications that prolong hospitalization following surgery occur in approximately 20% of patients (27-29). Since the long term effects of this operation on growth are unknown, gastric bypass surgery should currently be reserved for adolescents who have achieved or are nearing their adult height.

Tentative criteria for recommending gastric bypass surgery for obese adolescents are listed in Table 6. The likelihood of a spontaneous remission of obesity in adolescents who are in excess of 200% of ideal body weight (IBW) is extremely low (30). Furthermore, followup studies of obese adults indicate that adults obese as adolescents become even more obese as adults than adults with onset of obesity in adulthood (31). Among 25-34 year old men who weighed in excess of 200% IBW, an annual mortality of 3% per year has been observed (32). This group undoubtedly consisted of men obese during adolescence. For the obese adolescent who is 200% IBW, the risk of continuing disease appears to exceed the risk of surgery.

Gastric bypass is justified for the treatment of lesser degrees of obesity if the disease is accompanied by obstructive sleep apnea or primary alveolar hypoventilation. The mortality of these two aftereffects of obesity in children and adolescents is 25% (33). Severe hypertension attributable to obesity, or maturity onset diabetes mellitus are also indications, although the intervention is less urgent than if respiratory complications exist.

Table 6

Tentative Criteria for Surgical Treatment of Obesity in Adolescents.

1. Weight > 200% of ideal and/or
   major complication of the disease
   a. Primary alveolar hypoventilation
   b. Sleep apnea
   c. Persistent and severe hyptertension without other cause
   d. Maturity onset diabetes mellitus
2. Failure to lose weight under optimal conditions
   a. Restrictive dietary therapy
   b. Altered environment
   c. Family therapy

The illusions that patients undergoing bypass surgery will achieve ideal body weight, or need no further efforts at behavior modification must be dispelled prior to the operation. Among adults, mean weight losses after one year were 40 $\pm$ 15.5 kg, followed by a considerably smaller loss in the second postoperative year (25). Since gastric bypass depends for its effect on a feeling of satiety, and results in vomiting and abdominal pain when patients overeat, frequent or high caloric snacks will affect the ultimate result. Successful efforts to reduce these behaviors will promote further weight reduction.

In all cases, the recommendation for surgery should be reserved until after more conservative medical therapy has failed. Optimal treatment, based on the data available, consists of restrictive dietary therapy accompanied by vigorous family therapy. Depending on the circumstances, a trial of weight reduction in a hospital or foster home may be warranted. Success under such conditions may provide additional leverage to induce change within the family, and to avert the need for a surgical intervention.

Summary

Few reports deal quantitatively with the treatment of obesity in children and adolescents. The number of available therapeutic modalities is limited. Although studies comparing the particular value of one method with another are rare, studies of behavioral modification are among the more carefully controlled. However, weight losses observed in patients treated with behavioral modification to date have been no better than those observed in response to other modes of therapy. Evaluation of any clinical therapy among unselected patients is rare.

Despite the limited experience with family therapy, the association of variables within the family with the onset and course of the disease suggests that this mode of therapy deserves more intensive investigation. Gastric bypass should be reserved for adolescents in excess of 200% IBW, or with significant morbid effects of the disease, and only after optimal medical management has failed to induce weight reduction.

As indicated in Part 1 of this review, the frequency of spontaneous remissions decreases with advancing age and is lower among the more obese. Therefore, the success of therapy can only be determined by comparing remission rates among treated patients with the natural course of the disease in controls matched for age, sex, and severity. Whether age at onset (adipocyte hypothesis?), duration, or severity of obesity (synergy?), also affect the response to therapy

has never been tested in children or adolescents.

Our limited understanding of the determinants of the clinical course of obesity contributes to the limited success of therapy. Whether the behavioral mechanisms perpetuating obesity differ from those that induce onset is unknown; without their identification, successful therapy and prolonged remission will continue to be adventitious.

TREATMENT OF THE COMMUNITY

Since reports of clinical management of children and adolescents are so few, the rare accounts of therapy directed at the community occasion no surprise. At least four reports of interventions for obesity within school systems have been published, and several other programs are worth considering.

A common approach in school systems has been to enroll obese children and adolescents in an extracurricular physical activity program (34-36). In two instances significantly smaller increases in triceps skinfolds (35) or body weight (35,36) occurred among males enrolled in the program, but weight loss did not occur. When external funding was terminated, at least one of the programs (35) was discontinued.

A second school-based program combined attempts to alter multiple food related behaviors with nutritional education and exercise (37). In contrast to programs that emphasized exercise alone, both weight loss and decreased triceps skinfold measurements were observed.

These four reports are informative for several reasons. First, despite limited success, they demonstrate the potential for intervention within schools. Second, the end of at least one of the programs, coincident with the termination of federal funding, illustrates the need to root any intervention firmly within the community political process to ensure its continuity.

Still another approach deserves attention. In the United States, the National School Lunch Program currently provides lunches to over 25 million school children, and breakfasts to over two million (38). Even in schools that do not provide a free lunch, it may be purchased. Attention to the quantity and quality of foods served in schools would promote both treatment and prevention of obesity.

All of these interventions have focused on treatment of selected populations rather than the general community; approaches to the latter have yet to be studied. Education deserves special comment. Although classical health education has recently been criticized as a general public policy (39), the use of specific educational measures to destigmatize the obese and to heighten awareness of the aftereffects of obesity deserves broader application. An alternative is to concentrate educational efforts on populations where lack of knowledge about obesity is clearly associated with increased frequency of the disease. Sound evaluation of the effect of such programs would at least quantify their value as community interventions.

PREVENTION

Obesity, other forms of malnutrition, and the infectious diseases share a high prevalence and a substantial morbidity dependent on region and environmental situation. Although proper therapy for undernutrition and infectious diseases often abates the disease, it is more costly than prevention and often fails to eliminate the associated morbid effects of the illness. Current therapy for obesity is ineffective, and prevention rudimentary. Nonetheless, general experience with preventive medicine suggests that prevention of obesity in children and adolescents may be more cost-effective than treatment.

Primary deterrents to development of rational preventive programs for obesity are that neither the causes of the disease,nor the host and environmental characteristics of persons at risk have been clearly defined. Until

these are specified, prevention stands little hope of success.

Only one group of investigators has reported the results of a preventive program in clinical practice (40). In that study, the prevalence of obesity was significantly lower among three year old children whose parents had received intensive dietary instruction beginning when their children were three months old. Unfortunately, the comparison group consisted of children born six years earlier and apparently not matched for parental obesity, birth order, socioeconomic status, education or ethnicity. Without adequately matched controls, claims of successful prevention remain unsubstantiated.

EVALUATION OF TREATMENT PROGRAMS

Weight reduction and maintenance of weight loss are primary criteria for evaluating the therapy of obese children and adolescents. These standards reflect broader goals; to an extent, weight reduction reflects successful intervention in the course of the disease, while weight maintenance after reduction implies effective identification and elimination of cause. In circumstances where causes of clinical course and causes of origin are similar, weight reduction and weight maintenance focus on common problems. Differences in causes of origin and course may explain the disparity so often observed between short term weight loss and long term remission.

Reasonable gauges for success include information on growth, length of therapy, and duration of existing obesity. Since children and adolescents ideally continue to grow during therapy, the index of success will include not only the amount of weight lost, but also weight changes in relation to height. Comparisons of weight loss in early and late phases of management, or measurement of rates of weight loss, control the effect of length of participation. Finally, the true outcome of therapy can only be measured by comparing observed results with the established natural history of the disease, especially where seasonal variations in prevalence occur.

Absolute weight losses in the obese child or adolescent are a reasonable means to judge the short term value of different methods of management. So few patients now achieve ideal body weight under ordinary circumstances that to judge therapy by such an exacting standard is impractical. Although rapid weight loss followed by weight maintenance implies a differences from slow, progressive and continuous weight loss, no information currently explains why such variations occur, or which implies the better prognosis.

To emphasize weight maintenance as a separate goal may be premature since short term success rates are so low. However, with anticipated progress in the management of obesity, weight maintenance deserves careful consideration. Until cause is identified, effective prevention of recurrent obesity is improbable. As indicated, the need is for much more information about both social or metabolic causality if effective weight maintenance is to be promoted adequately. These areas are deservedly the focus of current research.

CONCLUSION

Much of the existing clinical and experimental research on identification, causality, natural history and management of obesity in children and adolescents has been unsystematic and all too often poorly designed. However, based on what has been accomplished to date, it is now possible to formulate areas for further investigation and to begin a more rigorous and serious test of fresh hypotheses.

Reliable identification of obesity awaits several important advances. Anthropometric techniques must be improved to estimate reproducibly fat mass in the excessively obese, and to identify children and adolescents whose excessive adiposity is accompanied by significant increments in lean body mass. Obviously, these objectives require correlation with measurements of body composition

to ensure validity. To gain wide acceptability, the techniques must be inexpensive, easy to perform, and applicable to all ages.

A second aim is to substitute pathological for statistical criteria in the diagnosis of obesity. Identification of pathologic fatness depends on long term prospective field studies that demonstrate strong associations of adiposity with aftereffects; that document the temporal association of obesity with intercurrent or subsequent disease; and fulfill the other criteria essential to establish cause.

Such longitudinal field studies will also enhance understanding of natural history of obesity. To date, most epidemiologic investigations of obesity in children and adolescents have considered selected populations, and have examined persistence of obesity rather than incidence, remission, relapse or return. The latter data are vital to estimate accurately the risk of aftereffects, because in all likelihood susceptibility varies with age and the course of the disease.

Studies of the natural history of obesity logically include identification of both causes of origin and causes of clinical course. Both are likely to affect the severity, natural history, and aftereffects of obesity. Causes of origin require prospective study of obese persons and are essential for rational therapy of individuals or communities. Although hypothetical causes of course may derive from intensive study of clinic patients, they require confirmation through studies of populations to avoid selection bias and for a proper estimate of their importance. Since causes are often population specific, careful description of time, place, and person is essential.

Most of the available evidence suggests that the causes of obesity in children and adolsecents are more likely environmental than metabolic. The economic, ethnic, and familial correlates of obesity suggest further that causes are psychosocial rather than biophysical, and that they are behaviorally mediated. Effective prevention depends on identification and modification of the psychodynamic and behavioral mechanisms leading to increased energy intake, to reduced energy expenditure, or to combinations of the two.

Causes affecting the clinical course of obesity are probably more complex than those of origin. The likelihood of synergy implies causes that are metabolic as well as environmental. Identification of persons at risk for aftereffects, persistence and relapse, and clarification of synergistic mechanisms will sharpen the focus of therapeutic efforts. Ultimately, the costs and risks of therapy can only be justified by comparison with the cost and risks of continuing disease. At present, such information is sadly deficient.

The resolution of these several problems offers an opportunity to assess the comparative cost-effectiveness of treatment and prevention. Current therapy of obesity is notably unsuccessful. This recognition, as well as confirmed historical experience with the benefits of prevention for infectious disease and other forms of malnutrition, suggests that prevention be accorded a higher priority than it now receives.

The undue prevalence of obesity in the United States also suggests that therapeutic and preventive interventions should be directed at the community. Both are within the compass of experimental epidemiology. Adequate control populations are essential for precise tests of hypotheses. The contribution of potentially confounding variables, such as shifts in the economy that alter food consumption, or newly acquired access to exercise facilities, must be measured. For both prevention and treatment, success is best quantified by comparing observed results with the established natural history of the disease.

Epidemiology, as medical ecology, is clearly the cornerstone of these investigations. Obesity is a disease of populations as well as of indivudals within those populations. Knowledge of causality is essential to its diagnosis, natural history, treatment, and prevention. William Budd, describing his approach to typhoid fever over a century ago, stated the broad problem in succinct fashion (41): "To know how these powers take effect, in what way they grow to such a height, and to learn from these, perchance, by what means their

operation may be defeated, are problems in which human happiness is deeply interested".

ACKNOWLEDGEMENTS

I gratefully acknowledge the teaching and advice of Dr. John Gordon in the preparation of this review, and the review of preliminary drafts of the manuscript by Drs. Richard Grand, John Udall, Vernon Young and Nevin Scrimshaw. Dr. Barry Dym's perceptive work with families in our clinic has been instrumental to my understanding of the role of family dynamics in the onset and course of childhood obesity. The patient secretarial assistance from Ruth Urell and Priscilla Farry has been invaluable.

REFERENCES

1. COATES, T.J. and THORESON, C.E. Treating obesity in children and adolescents: a review. Am. J. Public Health, 68: 143-151, 1978.

2. RAYNES, P.H.W. and COURT, J.M. The effect of dietary restriction and anorectic drugs on linear growth velocity in childhood obesity. Postgrad. Med. J., 51 (Suppl. 1): 120-125, 1975.

3. AMERICAN ACADEMY OF PEDIATRICS COMMITTEE ON NUTRITION. Factors affecting food intake. Pediatrics, 33: 135-143, 1964.

4. HAMMAR, S.L., CAMPBELL, V. and WOOLEY, J. Treating adolescent obesity. Clin. Pediatr., 10: 46-52, 1971.

5. ALLEY, R.A., NARDUZZI, J.V., ROBBINS, T.J. WEIR, T.F., SABEH, G. AND DANOWSKI, T.S. Measuring success in the reduction of obesity in childhood. Clin. Pediatr. 7: 112-118, 1968.

6. NATHAN, S. and PISULO, D. Psychological observations of obese adolescents during starvation. J. Am. Acad. Child. Psych., 9: 722-740, 1971.

7. SASH, S.F. Treating obesity in schoolboys. S. Af. Med. J., 50: 797, 1976.

8. BROOK, C.G.D., LLOYD, J.K. and WOLFF, O.H.: Rapid weight loss in children. Brit. Med. J., 2: 44-45, 1974.

9. WOLFF, O.H. Obesity in childhood. Quart. J. Med., 24: 109-123, 1955.

10. YANG, M.H. and VANITALLIE, T.B. Composition of weight lost during short-term weight reduction. J. Clin. Invest., 58: 722-730, 1976.

11. MERRITT, R.J., BISTRIAN, B.R., BLACKBURN, G.L. and SUSKIND, R.M. Consequences of modified fasting in obese pediatric and adolescent patients. I. Protein-sparing modified fast. J. Pediatr., 96: 13-19, 1980.

12. MERRITT, R.J. Therapy of pediatric obesity: effectiveness of current treatment and evaluation of protein supplemented fasting. Doctoral thesis. Massachusetts Institute of Technology, 1977.

13. BROWNELL, K.D. and STUNKARD, A.J. Behavioral treatment of obesity in children. Am. J. Dis. Child., 132: 403-412, 1978.

14. RIVINUS, T.M., DRUMMOND, T. and COMBRINCK-GRAHAM, L. A group behavior treatment program for overweight children: results of a pilot study. Pediatr. Adolescent Endocrinol. 1: 55-61, 1976.

15. ARAGONA, J., CASSADY, J. and DRABMAN, R.S. Treating overweight children through parental training and contingency contrasting. J. Appl. Behav. Anal., 8: 269-278, 1975.

16. HEYDEN, S., DEMARIA W., BARBEE, S. and MORRIS, M. Weight reduction in adolescents. Nutr. Metabol., 15: 295-304, 1973.

17. GROSS, I., WHEELER, M. and HESS, K. Treatment of obesity in adolescents using behavioral self-control. Clin. Pediatr., 15: 920-924, 1976.

18. WHEELER, M.E. and HESS, K.W. Treatment of juvenile obesity by successive approximation control of eating. J. Behav. Ther. Exp. Psychiat., 7: 253-241, 1976.

19. STANLEY, E.J., GLASER, H.H., LEVIN, D.G., ADAMS, P.A., and CALEY, I.L. Overcoming obesity in adolescents. Clin. Pediatr., 9: 29-86, 1970.

20. MASSENGALE, O.N. The obese adolescent. Clin. Pediatr., 4: 649-654, 1965.

21. ANDELMAN, M.B., JONES, C. and NATHAN, S. Treatment of obesity in underpriviledged adolescents. Clin. Pediatr., 6: 327-330, 1967.

22. SEATON, D.A. and ROSE, K. Defaulters from a weight reduction clinic. J. Chron. Dis., 18: 1007-1011, 1965.

23. STUNKARD, A. and MCLAREN-HUME, M. Results of treatment for obesity. Archiv. Int. Med., 103- 79-85, 1959.

24. FIKRI, E. and CASSEL A, R.R.: Jejunoileal bypass for morbid obesity. Ann. Surg., 179: 460-464, 1974.

25. MASON, E.E., PRINTEN, K.J., BLOMMERS, T.L., LEWIS, J.W., and SCOTT, D.H. Gastric bypass in morbid obesity. Am. J. Clin. Nutr., 33: 395-405, 1980.

26. SOPER, R.T., MASON, E.E., PRINTEN, K.J. and ZELLWEGER, H. Gastric bypass for morbid obesity in children and adolescents. J. Pediatr. Surg., 10: 51-58, 1975.

27. GRIFFEN, W.O., JR. Gastric bypass for morbid obesity. Surg. Clin. N. Am., 59: 1103-1112, 1979.

28. BUCKWALTER, J.A. and HERBST, C.A., JR. Complications of gastric bypass for morbid obesity. Am. J. Surg., 139: 55-59, 1980.

29. GOMEZ, C.A. Gastroplasty in morbid obesity. Surg. Clin. N. Am., 59: 1113-1120, 1979.

30. BORJESSON, M. Overweight children. Acta. Paediatr. Scand., 51: Suppl. 132, 1962.

31. RIMM, I.J. and RIMM, A.A. Association between juvenile onset obesity and severe adult obesity in 73,532 women. Am. J. Pub. Health, 66: 479-481, 1976.

32. DRENICK, E.J., BALE, G.S., SELTZER, F. and JOHNSON, D.G. Excessive mortality and causes of death in morbidly obese men. J. Am. Med. Assoc., 243: 443-445, 1980.

33. RILEY, D.J., SANTIAGO, T.C. and EDELMAN, N.H. Complications of obesity - hypoventilation syndrome in childhood. Am. J. Dis. Child., 130: 671-674, 1976.

34. CHRISTAKIS, G., SAJECKI, S., HILLMAN, R.W., MILLER, E., BLUMENTHAL, S. and ARCHER, M. Effect of a combined nutrition education and physical fitness program on the weight status of obese high school boyd. Fed. Proc., 25: 15-19, 1966.

35. SELTZER, C.C. and MAYER, J. An effective weight control program in a public school system. Am. J. Pub. Health, 60: 679-689, 1970.

36. JETTÉ, M., BARRY, W. and PEARLMAN, L. The effects of an extracurricular activity program on obese adolescents. Canad. J. Pub. Health., 68: 39-42, 1977.

37. BOTVIN, G.J., CANTLON, A., CARTER, B.J. and WILLIAMS, C.L. Reducing adolescent obesity through a school health program. J. Pediatr., 95: 1060-1062, 1979.

38. EGAN, M.C. Federal nutrition programs for children. Pediatr. Clin. N. Am., 24: 229-239, 1977

39. COHEN, C.I. and COHEN E.J. Health education: panacea, pernicians or pointless? N. Engl. J. Med., 299: 718-720, 1978.

40. PISACANO, J.C., LICHTER, H., RITTER, J. and SIEGAL, A.P. An attempt at prevention of obesity in infancy. Pediatrics, 61: 360-364, 1978.

41. BUDD, W. Typhoid Fever. New York, George Grady Press, 1931.

NUTRITION RESEARCH, Vol. 1, pp.303-309, 1981
0271-5317/81/030303-07$02.00/0 Printed in the USA.
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SUMMARIES OF CONFERENCES

Society for Pediatric Research

The annual meeting of the Society was held in San Francisco April 27-April 30, 1981. In the summaries of selected presentations that follow, the abstract number (published in Pediatric Research 15: 440-734, 1981) is given in parenthesis following the name of the chief author.

Studies of the interaction of infant feeding and growth has continued to be a productive area of study for pediatricians. In fact, as J. BERNBAUM and co-workers reported (1247) non-nutritive sucking alone in small premature effects a significant reduction in the time of feeding, a shorter transition to total post-operative feeding, a reduced intestinal transit time, and a greater percent weight gain per week, despite an identical caloric intake compared to other gavage-fed infants. Once the child is on oral intake, S.J. Gross reported (559) that breast milk obtained from donors following pre-term delivery induced a better weight gain than milk obtained from donors three months post-partum. Infants fed pre-term breast milk demonstrated similar growth and metabolic responses to infants fed cow's milk formula.

PENCHARZ and co-workers (620) further showed that rates of protein flux, whole body protein synthesis, and catabolism were all significantly higher among 30 pre-term infants fed breast-milk. These results are consistent with increased recycling of endogenous protein and amino acids, and suggest that the level of protein in term breast milk may be only marginally adequate for the growth of the premature infant. A second paper by the same group (1392) reported that small-for-gestational age (SGA) infants had rates of protein turnover, synthesis and catabolism that were 20-30% greater than those in normal premature infants. Similar nitrogen balance, and increased 3-methyl-histidine excretion in the SGA infants, suggested higher rates of muscle degradation, perhaps reflecting more rapid maturation and remodelling of muscle tissue. Another study from Toronto, by Chessex et al. (233), used indirect calorimetry to calculate the cost of growth of very low birthweight (VLBW) infants. There was a high, positive, correlation ($r = 0.86$) between metabolic rate and weight gain. Approximately 53% of energy intake was used for growth; 45% of intake was either the actual protein or energy stored, and 8% was for tissue synthesis. However, fat accretion was three times greater than that in the fetus. The authors commented further that although energy intake is lower among breast fed infants, rates of protein deposition are similar but fat deposition is in the range of intrauterine fat accretion. In a subsequent poster session (531), this same group demonstrated a significant interrelationship between age, energy intake, metabolic rate and weight gain.

With regard to supplemental feeding of low birth weight infants two relevant papers were presented. VILEISIS and others (1457) compared the effects of triglyceride infusions (Intralipid) on glucose levels in two groups of pre-

mature infants. Group A was given 0.25 gm/kg/d, and group B 0.5gm/kg/d. Although glucose levels were comparable in the two groups, group B had a significantly greater increase in glucose above baseline levels. One potential mechanism is that Intralipid may drive gluconeogenesis by increasing the available supply of glycerol. Intralipid may also reduce the killing potential of polymorphonuclear leukocytes by acting as a particulate stimulus to phagocytosis and superoxide production thereby exhausting substrate available for these reactions (Cleary, et al, 533). The in vitro correlate of this phenomenon is not clear.

Two papers considered the possibility that the fetal alcohol syndrome (FAS) was a consequence of intrauterine undernutrition. Fisher et al. (549) recognized that the microcephaly, LBW, and reduced length of FAS infants was consistent with fetal undernutrition, and showed that high doses of alcohol (2, 5, 10 and 20 μM) progressively inhibited amino acid uptake. Since these doses were 10^3 times those found in humans after alcohol ingestion, the physiologic consequence of their observations is not clear. Ghishan et al. (554) suggested that zinc deficiency might be implicated in the FAS. Studies of pregnant rats given alcohol reduced placental zinc uptake by 40%; chronic alcohol ingestion resulted in decreased fetal weights, reduced total body zinc and diminished zinc uptake as well.

Both Saudubray et al. (1181) and Wolfsdorf et al. (1191) studied the metabolic response of normal children to a 24-hour fast. In both studies, ketone levels were inversely related to age, and levels at the end of 24 hours were higher than those observed in adults fasted for one week. No differences were seen in the ketotic response of children with ketotic hypoglycemia. Both papers emphasized the need to consider age in the assessment of the ketotic response.

Several papers on obesity indicated the increased attention this highly prevalent disorder has begun to receive. EPSTEIN and co-workers (47) studied the effect of targeting the obese child and obese parent (Group I) only the obese child (Group II), or specifying neither (Group III) for therapy. Children in two parent families in which at least one parent was obese were treated. Although no significant differences in weight loss occurred, significantly more children from group I maintained their losses. As compliance increases, more attention should logically focus on diet. As Dietz et al. (540) found, the inclusion of 1gm/ kg IBW/d of glucose with 1.5gm/kg IBW/d of protein appeared more optimal for protein metabolism than an isocaloric, isonitrogenous diet containing fat. Glucose metabolism, measured directly using a primed constant infusion of 1-^{13}C-glucose, was directly related to protein catabolism but only in the absence of carbohydrate; these data suggested that the interrelationship of glucose and protein metabolism was strongly dependent on the diet considered. Although the failure to achieve a plateau in nitrogen losses occurred in three of nine adolescents studied on carbohydrate free diets, Archibald et al. (2), using 2.5 gm protein/kg IBW/d, did not observe this phenomenen in a study of 17 obese adolescents. Of concern in this latter study was that the loss of body potassium appeared bipnasic, despite adequate supplements of potassium. One of the long-term complications of obesity, the effect of diabetes on the outcome of pregnancy, may be significantly improved by the use of an insulin pump during pregnancy according to RUDOLF and co-workers (1408). Improved control of blood glucose, reduced fluctuations of glucose and normal levels of glycosylated hemoglobin were shown in seven class C to FR pregnant diabetics treated by insulin pump therapy. Their infants had no macrosomid, normal skinfolds, no hypoglycemia, respiratory distress syndrome, jaundice or polycythemia. Such therapy promises improved outcomes for infants of diabetic mothers.

More fuel for the controversy regarding iron and infection was presented by Pearson et al. (849). Although they found the addition of iron to normal heat-treated serum abolished the bacteriostatic properties of that serum, they observed no differences in the ability of three iron deficient, 17 normal, or 13 iron overloaded sera to support the growth of two pathogenic E. coli strains in vitro. Therefore the effect of adding iron to sera cannot be replicated by normal physiologic sera, perhaps because added iron is more available for bacterial growth. These data do not support the role of iron in enhancing susceptibility to infection.

When does iron deficiency have pathologic consequences? Before the hemoglobin falls, according to OSKI and co-workers (846). In this study a larger group of 9-13 month old infants were tested with the Bayley test for mental development, all of whom had hemoglobins above 11.0 gm/dl. Infants were divided into four categories based on ferritin, free erythrocyte protoporphyrin, and mean corpuscular volume. After initial testing, each infant was treated with intramuscular iron and retested one week later. Scores rose significantly higher among the two groups with a high FEP, with or without a low MCV, suggesting strongly that iron deficiency without anemia was accompanied by behavioral change.

According to Greer et al. (557), bone mineral content measured by photon absorptiometry, and serum levels of 25-OH vitamin D were decreased in six month-old breast fed infants whose mothers did not receive supplemental vitamin D. After the study was unblinded at six months, all breast fed infants received 400 IU/d of vitamin D supplements and by 12 months of age, no differences were observed. The relationship of serum D levels to hypocalcemia in infants of diabetic mothers was investigated by STEICHEN and co-workers (1440). The observation of comparable levels of 25-OH vitamin D and 1,25 OH-vitamin D in hypocalcemic and normocalcemic infants, and the significantly increased prevalence of hypocalcemia in infants whose mothers were in poor diabetic control during pregnancy suggested that diabetic control rather than vitamin D metabolism was the major factor contributing to hypocalcemia.

Continued controversy on the antioxidant role of vitamin E in oxygen-induced lung damage was provided by HANSEN and BLAND (1319). Vitamin E provided no protective effect on either lung fluid filtration or microvascular permeability to protein in lambs, despite a ten-fold increase in plasma tocopheral levels.

A report of carnitine deficiency in a pair of twins was presented by KERR and co-workers (1142). Both presented with fatty liver, transient erythroblastopenia, hypoglycemia and cardiomyopathy. After finding low levels of carnitine in the tissue of one of the pair who died at age 7 months, low levels of carnitine were also found in the plasma, muscle, and liver of the surviving twin. An RQ prior to carnitine therapy was 0.94, and after carnitine administration was 0.78 suggesting that increased glucose utilization induced hypoglycemia. Carnitine administration improved muscle, hepatic, and cardiac function.

Finally, two papers considered specific nutritional problems in cystic fibrosis. Studies by ISENBERG and POWELL (566) showed that despite normal fat digestion, absorption of free fatty acids was still impaired. Micellar incorporation of free fatty acids appeared reduced although bile acid concentrations and intraluminal pH were normal. According to Harper et al. (1663) essential fatty acid deficiency in rabbits impaired macrophage lysozyme production and intracellular killing of Staphylococcus aureus and Pseuodomonas aeruginosa. These results suggested that essential fatty acid deficiency could

contribute to impaired lung defense mechanisms in cystic fibrosis.

W. H. DIETZ, JR.

American Federation for Clinical Research

The clinical meetings were held in San Francisco April 24-28, 1981. This brought together members of the American Society for Clinical Nutrition, the American Federation for Clinical Research, the American Society for Clinical Investigation, the American Association of Physicians and the Society for Investigative Dermatology in individual and joint plenary and subspecialty sessions. Selected short scientific papers in nutrition were presented in the ASCN meeting, and the combined ASCN/AFCR/AAP sessions had a number of papers of nutritional interest as well.

The winner of the 1981 ASCN Medical Student Research Award was CHARLES L. BAUM of the Pritzker School of Medicine, University of Chicago. Working in I.H. ROSENBERG'S laboratory, BAUM studied the Hydrolysis of Nicotinamide Adenine Dinucleotide by Intestinal Brush Border Membranes. The research concluded that the food form of niacin, NAD, was hydrolyzed prior to intestinal uptake by phosphorylase enzymes in the brush border.

Two papers presented exciting innovative news of value to all clinical nutritionists as they represented emerging topics. N.N. ABUMARD reported on a case of Amino Acid Intolerance During Prolonged Total Parenteral Nutrition Reversed by Molybdenum. The patient showed low urinary and serum levels of uric acid and elevation of circulating methionine responsive to supplementation with ammonium molybdate. The clinical and biochemical pattern was ascribed to decreased activity of xanthin oxidase and sulfite oxidase, the two molybdenometalloenzymes in mammals. This is the first time a report of putative human molybdenum deficiency had been presented to a forum of nutritionists. These findings may explain some additional features of TPN-induced abnormalities in man. That some of the reliance on TPN may be shifted to an enteral route even in patients with severely compromised intestinal function was indicated by H.L. GREEN of Vanderbilt in his paper on Nasogastric Tube Feeding at Home: A Method for Adjunctive Nutritional Support of Malnourished Patients. His patients were maintained at home, and most gained or maintained body weight. The treatment was even successful in patients with imflammatory bowel disease in which "bowel rest" has been considered to be a necessary concomitant of intensive nutritional repletion. The experience with tube feeding in IBD patients at the University of Chicago has also been encouraging, I.H. ROSENBERG reported in the discussion. The enteral route certainly provides more safety, versatility and economy than the parenteral alternative. More will certainly be heard from the home enteral nutrition experience.

Elsewhere among the program of scientific papers, a number of presentations focused on TPN. M.E. BURT from the NIH reported data on the Effect of Total Parenteral Nutrition on Protein Metabolism in Man. In this instance, patients with localized, obstructive esophageal cancer - receiving only radiation therapy appeared to maintain weight and body composition. Endogenous insulin appeared to facilitate maintenance of nitrogen metabolism. M. SHIKE of Toronto reported another experience of TPN in patients with malignancy in the paper Nutritional Status of Patients with Lung Carcinoma: The Effect of Chemotherapy and Parenteral Nutrition. Patients on TPN during a four week period of chemotherapy gained more weight, exclusively fat, than orally-fed patients. This

suggested to the authors that small cell carcinoma of the lung produces a hypermetabolic (but not protein wasting) state which cannot be met by spontaneous caloric intake. The unusual trial of short-bowel syndrome, antibiotic therapy and a decade of home parenteral nutrition provides a setting in which R.C. BOZIAN of Cincinnati described Biotin Deficiency During Prolonged, Home Total Parenteral Nutrition. A biotin-responsive exanthem and buccal lesion developed in their patient. The valididy of endogenous biotin production in the colon is supported by this demonstration. The authors suggest the inclusion of biotin in vitamin formulations for long-term TPN. Finally, from Atlanta, D. RUDMAN in a paper on Requirements for Cystine and Tyrosine in Cirrhosis, provided insight into the capacity of conventional amino acid mixtures used in TPN to support N retention in severely decompensated cirrhosis. Commercial TPN solutions omit the highly insoluble tyrosine and cystine on the assumption that they will be produced as needed by hepatic metabolism of phenylalanine and methionine. Abnormal plasma aminograms and negative N balance despite TPN were observed in a group of cirrhotic patients with severe hepatic dysfunction. These were normalized by the provision and doses of cystine and tyrosine.

A concern with obesity and its treatment was an original tenet of clinical nutrition as a medical subspecialty. Active interest in issues of energy regulation and adipose accumulation continues. Two papers from the laboratory of F.X. PI-SUNYER bear on these issues. In the presentation on the Effect of Excise on Spontaneous Calorie Intake in Obesity, R. WOO was unable to demonstrate a compensatory increase in food intake of obese women (mean of 167% ideal weight) when mild and moderate increases in energy expenditure were induced with treadmill exercises. A negative energy balance of over 400 kcal/day was maintained throughout a 19-day period of moderately augmented energy output. F.X. PI-SUNYER presented a paper on Cholecystokin Octapeptide (CCK-8) Decreases Food Intake in Obese Men in which an infusion of saline or CCK-8 was given to obese men (means 138% ideal weight) in a double blind fashion during a standard liquid breakfast. The octapeptide decreased intake by an average of 112 kcal, primarily due to an earlier cessation of food consumption. WILLIAM H. DIETZ, JR. of M.I.T. presented a paper on Hypocaloric Diets in Obese Adolescents: Effects of Dietary Glucose or Fat on the Interrelationship of Protein and Glucose Metabolism in which data on hepatic glucose production and nitrogen balance in nine obese adolescents consuming a protein plus fat or protein plus glucose diet (isocaloric) were reported. Cumulative nitrogen balance was better on the protein plus carbohydrate diet. Innovative methodology including the use of stable isotope-labelled substrates ($H_2{}^{18}O$ to determine body composition; ^{13}C-glucose to measure glucose oxidation) was an excitory feature of this work. N. BABA of Columbia University presented Enhanced Thermogenesis and Diminished Deposition of Fat in Response to Overfeeding with Diet Containing Medium Chain Triglyceride. In a gastrostemized rat model, overfeeding MCT produced smaller weight increases, small adipocytes, and increased oxygen consumption and BMR as compared to long chain triglycerides. Increased thermogenesis on the MCT diet explained the decreased fat composition.

Vitamin D commanded attention at the San Francisco meetings in both clinical nutrition and metabolism sessions. The intestinal metabolism of the vitamin was covered in two papers from I.H. ROSENBERG'S laboratory in Chicago. K.L. POLLACK, an undergraduate student, presented a paper entitled Chylomicron-Dependent Absorption and Transport of Vitamin D and 25-Hydroxy-Vitamin D in the Rat, while his co-worker, M.D. SITRIN presented an extension of this work Influence of Bile Salts and Fat on Intestinal Absorption of Vitamin D and 25-Hydroxy-vitamin D in the Rat. Both studies employed cannulation of the bile and thoracic ducts and intestinal instillation of radio-labelled vitamins; both demonstrated a less efficient absorption of vitamin D than 25-OH-vitamin D and

a predominant uptake into the portal blood as compared to the lymphatic drainage. This observation might explain the relatively lesser expression of osteomalacia as compared to other manifestations of fat-soluble vitamin deficiencies in abetalipoproteinemic patients.

Important new findings related to vitamin D in human nutrition were presented. Two presentations from the Massachusetts General Hospital addressed the issue of vitamin D nutrition in the aged. M. HOLICK discussed Aging Significantly Decreases the Capacity of Human Epidermis to Produce Vitamin D, while D. SLOVICK presented Deficient 1,α25-$(OH)_2$-vitamin D Production in Osteoporotic Patients. The pool of F-dehydrocholesterol the provitamin D, in human skin was reduced in skin biopsies from older subjects as compared to younger volunteers, and equivalent ultraviolet treatment produced less vitamin D in the aged sample. Renal production of 1α,25 dihydroxyvitamin D during a 24-h infusion of the 1-34 synthetic peptide fragment of parathyroid hormone was reduced in aged subjects with osteoporosis (mean age, 59 years) as compared with a group of younger subjects (mean age, 29 years). The lack of age-matched controls in the latter study makes any conclusion about the dominance of age versus bone disease tendency in the renal hyporesponsiveness less valid. A group of Army researchers, which included the late ROBERT HERMAN, also provided new insights into hormonal responses and bone metabolism. The paper, presented by D.D. BIKLE and entitled Cyclic Nucleotide and Bone Mineral Responses to Dietary Calcium by Subjects with Osteoporosis and Hyperparathyroidism, suggested a hyperresponsiveness to endogenous regulators of the cyclic nucleotides (cAMP, cGMP) of the kidney in four post-menopausal osteoporotic subjects. The study manipulated daily calcium intake (100 mg vs. 1000 mg). Unfortunately, control and hyperparathyroid subjects were not matched for age or sex.

Trace mineral nutrition was strongly represented in the scientific programs. J. COOK of the University of Kansas developed a Rapid Qualitative Assessment of Iron Status for field use which requires no laboratory equipment, but, by visual inspection, permits differences in serum ferritin concentrations representing iron deficiency, iron adequacy, and iron overload to be distinguished. Clinical evaluation of zinc nutrition may be facilitated by the data presented by A.S. PRASAD of Detroit on Nucleoside Phosphorylase in Zinc Deficiency. The activity of this enzyme was reduced in the red cells of zinc deficient rats as compared to their pair-fed controls and in a patient followed prospectively through zinc depletion and repletion. Two additional studies from Detroit looked at zinc status in uremic patients on hemodialysis. In a paper presented by W.A. BRIGGS, Mononuclear and Polymorphonuclear Cell Function in Zinc Deficient Hemodialysis Patients, an effect of zinc deficiency on the chemotatic function was reported. In a paper Zinc Deficiency in Uremia: Response to Oral Zinc Supplementation S.K. MAHAJAN showed that a daily dose of supplemental zinc administered to patients with uremia could improve indices of zinc nutrition including plasma zinc, leukocyte zinc and serum RNAase. This suggested that zinc depletion is, indeed, common in uremic patients undergoing hemodialysis.

The symposium on The Biological Availability of Mineral and Trace Mineral from Food co-chaired by I.H. ROSENBERG and N.W. SOLOMONS, included discussion of calcium (L.H. ALLEN), selenium (V.R. YOUNG), zinc (N.W. SOLOMONS) and copper (H.H. SANDSTEAD). A pre-meeting workshop on the complementary theme Mineral and Trace Mineral Absorption in Man was organized by the ASCN Committee on Research. The ASCN Presidential Address was delivered by VICTOR HERBERT was entitled Will Questionable Nutrition Overwhelm Nutritional Science? It was an impassioned call for those who represent the community of nutritional scientists and scholars - adherents of premises of the experimental methods,

peer review, clinical trial - to confront and address the misinformation and open intimidation from those who promote questionable and unscientific nutritional beliefs and practices.

N.W. SOLOMONS

NUTRITION RESEARCH, Vol. 1, p. 311, 1981
0271-5317/81/030311-01$02.00/0 Printed in the USA.
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ANNOUNCEMENTS

Change in Editorial Office of NUTRITION RESEARCH

All manuscripts, books for review and editorial correspondence should henceforth be sent to Dr. R.K. Chandra, Editor-in-Chief, Nutrition Research, Janeway Child Health Centre, St. John's, Newfoundland, A1A 1R8, Canada. Telephone (709) 778-4519/4520.

European Society of Parenteral and Enteral Nutrition

The Third Congress of the European Society of Parenteral and Enteral Nutrition will take place in Maastricht, The Netherlands, September 27-30, 1981. For further information contact: Congress Secretariat, 3rd European Congress on Parenteral and Enteral Nutrition, c/o Department of Surgery, University of Limburg, St. Annadal Hospital, Maastricht, The Netherlands.

Regional Workshops in Clinical Nutrition

The American Society for Clinical Nutrition (ASCN) will cosupport and cosponsor regional workshops in clinical nutrition. The objective of the program is to disseminate clinical nutrition information to practicing physicians through regional workshops to be organized by individual Society members in conjunction with institutional postgraduate education programs. Up to $1,000 per workshop is available for use only to reimburse outside speakers for their travel costs and to provide appropriate honoraria for them. Applications may be submitted at any time, and selections are made on a quarterly basis; April 1, July 1, October 1, and January 1 for meetings scheduled in the immediately-following quarter. Applications must be submitted in twelve copies, and provide the following information. Date, topic, and faculty listing; the expected goals of the workshop; the number of medical practitioners expected in the audience; anticipated budget, to include local support for the workshop, other sources of support, anticipated expenses, and the estimated cost for travel and honoraria for outside speakers; and a list of outside speakers with a brief description of background and particular expertise to speak on the subject indicated, along with the estimated cost for travel and honorarium for each.

For further information write to Regional Workshop Program, American Society for Clinical Nutrition, 9650 Rockville Pike, Bethseda, MD 20014.

NUTRITION RESEARCH, Vol. 1, pp. 313-314, 1981
0271-5317/81/040313-02$02.00/0 Printed in the USA.
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EDITORIAL

Pathogenesis of cell-mediated immunity deficiency in malnutrition

The interaction between our daily bread and ourselves may underlie many disease states. One example of the important influence of nutrition on health is susceptibility to infectious illness. The pattern of infections encountered in malnourished individuals suggests that protein-energy deficiency results in depressed cell-mediated immunity responses. The microorganisms commonly isolated from such undernourished patients include Klebsiella, Giardia lamblia, Pneumocystis carinii, and Candida albicans. Systematic investigations of host immune responses have shown a consistent impairment of cellular immunity(1-7). Human studies have demonstrated lymphopenia, depressed delayed cutaneous hypersensitivity reactions, lower numbers of rosette-forming T (thymus-dependent) lymphocytes, decreased lymphocyte proliferation response to mitogens and antigens, and occasionally, reduction in the release of lymphokines. The pathogenetic mechanisms underlying impaired cell-mediated immunity have not been clearly defined. Reduction in the number of mature functional T cells is an important factor; changes in cell number may be brought about by reduced proliferation, cell lysis, sequesteration, or lack of maturation from precursor stages. There is some evidence that many of these factors are operating in the malnourished individuals. For instance, reduction in the activity of thymic hormone(s) has been demontsrated in the sera of children with marasmus and kwashiorkor.Moreover, incubation of mononuclear cell preprations from these children in the presence of thymopoietin increases the ability to form rosettes with sheep red blood cells. Elevation of leukocyte deoxynucleotidyl transferase in malnutrition is an additional indirect evidence for disturbed maturation of T cell precursors.

The limitations of obtaining data in man have led to many excellent studies investigating the cellular interactions involved in immunodeficiency seen in malnutrition. The role of T cell subpopulations and macrophages is only now beginning to be looked at. One such attempt is being reported by McMurray and colleagues in this number of Nutrition Research. Within the limitations of the study design, the report assesses the role of macrophages in cell-mediated immune responses of malnourished guinea pigs. It must be remembered, however, that the immune response is a very complicated network, and dissection of one of its facet can give only partial answers. Results obtained in one species may not be valid for another. The severity of protein deficit is an important determinant of the immunologic changes observed; occasionally, experiments may reveal completely opposing results depending upon the protein content of animal diet. Finally, the most important test of altered immunity must remain the response to infectious challenge and final outcome in terms of both morbidity and mortality. This is true of man as well as laboratory animals.

R. K. Chandra

1. CHANDRA, R.K. Immunology of Nutritional Disorders. Arnold, London, 1980.

2. CHANDRA, R.K. and NEWBERNE, P.M. Nutrition, Immunity and Infection : Mechanisms of Interactions. Plenum, New York, 1977.

3. DREIZEN, S. Nutrition and the immune response. Int. J. Vitam. Nutr. Res. 40: 220-228, 1978.

4. GROSS, R.L. and NEWBERNE. Role of nutrition in immunologic function. Physiol. Rev. 60: 188-197, 1980.

5. SUSKIND, R.M. (ed.) Malnutrition and the Immune Response. Raven, New York, 1977.

6. CHANDRA, R.K. Immunodeficiency in undernutrition and overnutrition. Nutr. Rev. 39: 225-231, 1981.

7. CHANDRA, R.K. Nutritional deficiency. In: Immunodeficiency Disorders. R.K. Chandra (ed). Churchill Livingstone, Edinburgh, 1981 (in press).

NUTRITION RESEARCH, Vol. 1, pp. 315-325, 1981
0271-5317/81/040315-11$02.00/0
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EFFECT OF DIETARY CALCIUM AND PHOSPHORUS LEVELS ON THE UTILIZATION OF CALCIUM, PHOSPHORUS, MAGNESIUM, MANGANESE, AND SELENIUM BY ADULT MALES[1,2]

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ABSTRACT

The effects of dietary calcium and phosphorus levels on the utilization of phosphorus, calcium, magnesium, manganese, and selenium by nine adult males were examined during a 39-day balance study. The dietary treatments were a moderate calcium-moderate phosphorus diet (MCaMP), a moderate calcium-high phosphorus diet (MCaHP), and a high calcium-high phosphorus diet (HCaHP). The moderate and high levels of phosphorus were 843 and 2443 mg daily, respecitvely; the moderate and high levels of calcium were 780 and 2382 mg daily, respectively. Subjects were unable to maintain positive balance in regard to phosphorus when fed the moderate level of phosphorus (MCaMP) but maintained positive balance in regard to phosphorus when fed the high level of phosphorus (MCaHP, HCaHP). The addition of more calcium to the high phosphorus diets resulted in significantly greater excretion of phosphorus in the feces but significantly less excretion of phosphorus in the urine by subjects. The dietary treatments affected urinary excretion of calcium, magnesium, and selenium significantly, but the effects were small or were compensated for by changes in fecal excretion of the minerals. Thus the dietary treatments had no significant effects on overall retention of calcium, magnesium, manganese, and selenium by these subjects.

KEY WORDS: dietary phosphorus, dietary calcium, human balance study, magnesium, manganese, selenium

INTRODUCTION

A number of investigators have examined the effects of dietary calcium and phosphorus levels on the utilization of minerals by human subjects. Most nutritionists agree that subjects excrete less calcium in the urine when dietary phosphorus supplements are administered (1-8). However, some investigators have noted that the supplementation of diets with phosphorus did not improve overall calcium retention because changes in the absorption of calcium compensated for the decreased urinary calcium losses (1-4, 9); others, however, have observed no change in calcium absorption and thus a net improvement in calcium retention among subjects given phosphorus supplements (4-7).

[1]Research supported by College of Agricultural and Life Sciences, University of Wisconsin, Madison, WI, Project #2398 and by National Dairy Council.
[2]Part of the data in the paper was presented at the 65th annual meeting of the Federation of American Societies of Experimental Biology, Atlanta, GA, 1981 (Abstract #3994).

Similarly, dietary calcium supplements have been found to improve the phosphorus retention of some human subjects, particularly when dietary phosphorus levels are high (3,8). However, dietary calcium supplements usually have been found to increase fecal excretion and decrease urinary excretion of phosphorus by human subjects, without significantly affecting phosphorus retention (3,4,7,9,10).

These differences in the data may be due to differences in the age and gender of subjects or may be due to differences in the absolute and relative amounts of calcium, phosphorus and other components in diets fed subjects. High dietary levels of phosphorus, especially if the levels of calcium are low, have been shown to adversely influence bone metabolism in rats (11-14), dogs (15), mice (16), cats (17), and horses (18) but not in nonhuman primates (19). However, the rat, at least, may not be a good model for bone metabolism in humans (20). Thus the real significance of calcium to phosphorus ratios in human diets remains uncertain (21).

The dietary levels of calcium and phosphorus may affect also the metabolism of magnesium and some trace elements by men and animals. High levels of dietary calcium and phosphorus have been shown to increase the magnesium requirements of animals (22-24). Seelig (25) after a thorough review of the literature concluded that high dietary levels of calcium increased the magnesium requirements of human subjects. More recently Kim (7) found that high levels of dietary phosphorus decreased both apparent absorption and urinary excretion of magnesium by human subjects and thus did not affect overall retention of magnesium. Leichsenring et al. (26) noted that the addition of both calcium and phosphorus to human diets had no effect on overall magensium retention. However, Kim (7) noted that when subjects were fed high levels of both calcium and phosphorus they tended to retain less magnesium than when fed moderate levels of calcium and phoshporus.

Several investigators have examined the effect of dietary phosphorus and calcium on iron (27-29) and zinc (27,30,31) utilization by human subjects but very few investigators have examined the effects of dietary calcium and phosphorus on the utilization of other trace elements. High dietary levels of both calcium and phosphorus have been found to negatively affect manganese status of rats (32), chickens (33), and cattle (34). However, Spencer (30) found the manganese utilization of one subject was unaffected by variations in dietary phosphorus levels. Greger and Marcus (35) failed to observe any differences in selenium excretion by subjects when fed two levels of phosphorus.

The objectives of this study were to examine the effects of variations in calcium and phosphorus levels and ratios on the utilization of calcium, phosphorus, magnesium, manganese and selenium by human subjects.

METHODS

Experimental design. This 39-day metabolic study consisted of a 3-day orientation period followed by three 12-day experimental periods. The dietary treatments were a moderate calcium-moderate phosphorus diet (MCaMP), a moderate calcium-high phosphorus diet (MCaHP), and a high calcium-high phosphorus diet (HCaHP). The moderate and high levels of phosphorus were 843 mg and 2443 mg daily, respectively. The moderate and high levels of calcium were 780 mg and 2382 mg, respectively. Each subject participated in all three treatments. During each period three subjects were randomly assigned to each treatment.

Subjects. The nine male subjects ranged in age from 19.6 to 26.5 years. Their mean height was 179 ± 6 (S.D.) cm; their mean wieght was 72 ± 10 kg.

Subjects were healthy as determined by medical histories and examinations by a physician prior to the study.

Subjects were not confined within the laboratory during the study. They were required to consume all their meals at the metabolic facility during supervised time periods and to collect all excreta in designated containers. Subjects continued their normal routines as students during the study but refrained from unusually strenuous physical acitvity.

Subjects gave informed consent in a manner approved by a University of Wisconsin committee on the use of human subjects.

Dietary treatments. A limited, two-day cycle menu was fed throughout the study. The menus contained 250 g lemonade; 200 g frozen orange juice in menu 1 or 200 g canned apple juice in menu 2; 150 g Italian bread; 126 g individual apple turnover in menu 1 or 126 g individual cherry turnover in menu 2; 100 g ground beef (10% fat); 100 g canned applesauce in menu 1 or 100 g bottled grapefruit sections in menu 2; 100 g canned tomato juice; 100 g canned pineapple chunks; 100 g frozen blueberries in menu 1 or 100 g canned pears in menu 2; 100 g frozen wax beans in menu 1 or 100 g frozen green beans in menu 2; 50 g margarine; 60 g half and half; 60 g pound cake; 56 g jelly; 35 g potato chips in menu 1 or 45 g pretzels in menu 2; 40 g rice; 30 g dehydrated potato flakes; 30 g frozen onions; 30 g celery; 30 g frozen carrots; 30 g condensed cheddar cheese soup in menu 1 or 30 g condensed mushroom soup in menu 2; 280 g bottled lemon soda; 2 g salt; and 0.4 g pepper.

Foods were purchased in case lots to insure uniformity. All foods were prepared, cooked, and served with plastic and paper containers and utensils to prevent trace element contamination. Distilled-deionized water was used for food preparation and as a beverage by subjects.

The diets provided about 3050 kilocalories daily. Margarine, sugar, and candy were added to or reduced in diets as necessary to maintain the subjects' weights. The diets were calculated to provide 58 g protein daily and at least 100% of the Recommended Dietary Allowances (RDA) for iron, thiamin, niacin, vitamin A, and vitamin C and 83% of the RDA for riboflavin for 19 to 22 year old males (21).

The diet was supplemented daily with 400 IU vitamin D and 2 mg zinc as zinc sulfate. The zinc sulfate was dissolved in lemonade served to subjects at lunch. Tablets, which contained magnesium gluconate were consumed by subjects at each meal.

Subjects were given equal portions of calcium gluconate in solution with each meal to achieve the desired level of calcium in the diets. Subjects were given glycerol phosphate in gelatin capsules with each meal as part of the MCaHP and HCaHP treatments. They were given an equal number of empty gelatin capsules with each meal as part of the MCaMP treatment.

Composites of both menus were prepared three times prior to the study and three times during the study. The 12 food composites and all mineral supplements, caloric supplements, vitamin supplements, and gelatin capsules were analyzed for their mineral content (Table I).

Analyses. All food, fecal, and urine samples were collected and stored in acid-washed containers. Procedures to prevent trace element contamination were described previously (36).

TABLE I

Mineral Composition of the Diets

Mineral	Analyzed content of: Menu 1[1]	Menu 2[1]	Average Intake[2]: MCaMP	MCaHP	HCaHP
Calcium (mg)	391	366	780	780	2382
Phosphorus (mg)	853	836	843	2442	2443
Magnesium (mg)	270	240	364	364	364
Zinc (mg)	8.1	8.4	10.4	10.4	10.4
Manganese (mg)	3.17	3.48	3.35	3.37	3.40
Selenium (µg)	139	149	144	144	144

[1]Mean mineral content of diet composites (n=6).

[2]Average mineral intake of subjects from basal diet and from caloric, mineral and vitamin supplements.

Brilliant blue dye was administered to subjects before breakfast on days 5 and 11 of each period and feces were composited accordingly. Daily urine composites were made from 24 hour urine collections from each subject. Samples were acidified with hydrochloric acid and were frozen. Six-day urine composites were prepared from the daily composites.

Aliquots of food and fecal samples were dry ashed as described by Hegsted, et al. (5). They were analyzed for calcium, magnesium and manganese content by atomic absorption spectrophotometry and for phosphorus content by a colorimetric procedure (37). Known amounts of calcium, phosphours, magnesium and manganese were added to four food samples and processed in the same manner. The recoveries ranged from 100% to 102% for calcium, from 97% to 100% for phosphorus, from 94% to 98% for magnesium, and from 98% to 104% for manganese.

The calcium and magnesium content of diluted urine samples were determined directly by atomic absorption spectrophotometry. Lanthanum was added to urine samples and standards so that they contained 0.5% lanthanum. The concentration of manganese in the urine samples was too low (<0.02 mg/day) to be determined accurately by this procedure and hence were not reported in this paper. The phosphorus content of diluted urine samples were determined directly by a colorimetric procedure (37).

Food, fecal and urine samples were wet ashed with nitric acid, sulfuric acid, perchloric acid, and hydrogen peroxide and then analyzed for their selenium content by the fluorometric procedure described by Ch, et al. (38). Bovine liver standard, which was obtained fromthe National Bureau of Standard, was ashed and analyzed for selenium content in the same manner as the other samples five times. The analyzed selenium content of the samples was 1.0 ± 0.1 µg/g; the certified value was 1.1 ± 0.1 µg/g. Creatinine content of fresh urine samples was determined daily using an alkaline-picrate method (39).

The effects of the dietary treatments on fecal and urinary losses and balance were evaluated with analysis of variance in a randomized complete block

design (40). Means were then compared with Duncan's multiple range tests (40).

RESULTS

Subjects excreted significantly ($p<0.01$) different amounts of phosphorus in their feces when fed each of the three experimental diets (Table II). They excreted more phosphorus in the feces when fed the high rather than the moderate level of phosphorus. They excreted even more phosphorus in the feces when the dietary calcium level was increased from 780 to 2382 mg per day. Subjects apparently absorbed, on the average, 52%, 61% and 46% of the phosphorus in the MCaMP, MCaHP and HCaHP diets, respectively.

TABLE II

Mineral Excretion and Retention by Adult Males Fed Moderate and High Levels of Calcium and Phosphorus

	Treatments		
	MCaMP	MCaHP	HCaHP
Phosphorus (mg/day)			
fecal losses†	406 ± 56*[a]	957 ± 149[b]	1314 ± 200[c]
urinary losses†	481 ± 70[a]	1382 ± 138[c]	1062 ± 166[b]
apparent retention‡	-44 ± 86[a]	103 ± 138[b]	66 ± 117[b]
Calcium (mg/day)			
fecal losses†	677 ± 82[a]	689 ± 111[a]	2222 ± 267[b]
urinary losses†	240 ± 65[b]	147 ± 57[a]	212 ± 67[b]
apparent retention‡	-137 ± 59	-56 ± 80	-52 ± 244
Magnesium (mg/day)			
fecal losses†	209 ± 18[a]	241 ± 38[b]	239 ± 27[b]
urinary losses†	153 ± 28[b]	108 ± 19[a]	119 ± 24[a]
apparent retention‡	2 ± 17	15 ± 24	6 ± 21
Manganese (mg/day)			
fecal losses	3.57 ±0.31	3.46 ± 0.38	3.61 ± 0.33
apparent retention**	-0.22 ±0.31	-0.09 ± 0.40	-0.21 ± 0.33
Selenium (µg/day)			
fecal losses	37 ± 5	40 ± 5	38 ± 6
urinary losses#	57 ± 8[b]	56 ± 9[b]	52 ± 9[a]
apparent retention‡	49 ± 11	48 ± 12	54 ± 10

*mean ± S.D. (n=9)
‡apparent retention = intake - fecal losses - urinary losses
†means in row with different superscripts are significantly different at the $p<0.01$ level
#means in row with different superscripts are significantly different or at the $p<0.05$
**apparent retention = intake - fecal losses

Subjects excreted significantly ($p<0.01$) more phosphorus in their urine when fed the high phosphorus diets (MCaHP and HCaHP) than when fed the MCaMP diet. However, they excreted significantly less phosphorus in the urine when fed the HCaHP diet rather than the MCaHP diet.

All subjects, except two, were in negative phosphorus balance when fed 843 mg phosphorus daily, approximately the amount suggested in the Recommended Dietary Allowances. The average apparent retention of phosphorus by subjects was significantly ($p<0.05$) greater when they were fed the high level rather than the moderate level of phosphorus. The dietary calcium level did not significantly affect the overall rention of phosphorus.

Subjects lost significantly ($p<0.01$) more calcium in the feces when the dietary calcium level was increased from 780 mg to 2382 mg daily (Table II). Their mean apparent absorptions of calcium when fed the MCaMP, MCaHP and HCaHP diets were 13%, 12% and 7%, respectively.

The MCaMP and HCaHP diets provided different levels of dietary phosphorus and calcium, but each contained a 1:1 calcium:phosphorus ratio. Subjects excreted similar amounts of calcium in the urine when fed the two diets (MCaMP and HCaHP) with the 1:1 calcium:phosphorus ratios. They lost significantly ($p<0.01$) less calcium in the urine when they consumed the MCaHP diet, which contained a 1 to 3 calcium:phosphorus ratio, than when they consumed the other two diets.

All subjects were in negative calcium balance when they consumed the MCaMP diet. Neither the addition of calcium nor phosphorus to the diet resulted in a statistically significant improvement in calcium retention by the subjects. However, three subjects were in positive calcium balance when they consumed the MCaHP diet; five were in positive calcium balance when they consumed the HCaHP diet.

Subjects lost significantly ($p<0.01$) more magnesium in the feces but significantly ($p<0.01$) less magnesium in the urine when they consumed the high phosphorus diets (MCaHP and HCaHP) rather than the moderate phosphorus diet (MCaMP) (Table II). Thus the dietary treatments did not affect the subjects overall retention of magnesium.

The average apparent absorption of magnesium by subjects when fed the MCaMP, MCaHP, and HCaHP diets were 43%, 34% and 34%, respectively. Although the mean apparent retention of magnesium by subjects when fed each diet was positive, five subjects were in negative balance when they consumed the MCaMP diet; three were in negative balance when they consumed the MCaHP diet and two were in negative balance when they consumed the HCaHP diet. The apparent retention of magnesium by these subjects was statistically correlated to their retention of calcium ($r=0.587$, $p<0.05$), but not to their retention of phosphorus.

The dietary treatments did not affect fecal excretion or apparent retention of manganese by subjects (Table II). Six subjects appeared to be in negative balance in regard to manganese when fed the MCaMP and MCaHP diets; seven appeared to be in negative balance in regard to manganese when fed the HCaHP diet. Retention of manganese by these subjects was statistically correlated to their apparent retention of calcium ($r=0.615$, $p<0.05$) and magnesium ($r=0.730$, $p<0.05$).

The dietary treatments had no effect on fecal losses of selenium (Table II). The subjects, on the average, absorbed 74%, 72% and 73% of the selenium in the MCaMP, MCaHP and HCaHP diets, respectively.

Subjects lost significantly ($p<0.05$) less selenium in their urine when fed the HCaHP diet rather than the other two diets. The dietary treatments had no statistically significant effect on overall selenium retention by these adult males. All were in positive balance with regard to selenium throughout the study.

Urinary creatinine losses of each subject were constant throughout the study. The average (±S.D.) daily losses of creatinine by subjects when fed the MCaMP, MCaHP and HCaHP diets were 1.75 ± 0.30 g, 1.82 ± 0.26 g and 1.82 ± 0.29 g, respectively.

DISCUSSION

The level of phosphorus in the MCaMP diet fed to subjects in this study was approximately the amount specified in the R.D.A. for phosphorus (800 mg daily). Seven of the nine subjects were in negative balance with respect to phosphorus when fed the MCaMP diet. Other investigators also have noted recently that young adult males did not achieve positive balance in regard to phosphorus during short-term balance studies when fed 800 mg phosphorus daily (7,8). However, subjects fed very low levels of phosphorus have been found to adapt to diets which contain very low levels (<100 mg/day) of phosphorus by reducing urinary phosphorus excretion (41). It is not clear why subjects in this and previous studies (7,8) were unable to conserve sufficient phosphorus in this manner to achieve positive balance in regard to phosphorus within two weeks after the initiation of diets with moderate (~800 mg daily) levels of phosphorus.

The average adult male (35-50 years) who participated in the 1977-1978 Nationwide Food Consumption Survey consumed 0.8 g calcium and 1.4 g phosphorus daily (42). This is a phosphorus:calcium ratio (Ca:P) of 1:1.82. Some Americans are believed to consume more than 2.0 g phosphorus daily (28,43). These individuals could consume diets that contain Ca:P ratios of 1:3. Thus the MCaHP diet in this study was formulated to contain 2.4 g phosphorus and 0.8 g calcium daily. The Ca:P ratio was 1:3. When subjects consumed the MCaHP diet, they retained more phosphorus than when they consumed the MCaMP diet. However, the high level of dietary phosphorus and the low dietary Ca:P ratio did not adversely affect the overall retention of calcium, magnesium, manganese and selenium. Similarly other investigators have observed that the addition of just phosphorus (1-2 g) to the diets of human subjects did not depress overall retention of magnesium (1,7), manganese (30) and selenium (35). If the absolute level of calcium in the diets, the physiological state of the subjects and the forms of phosphorus supplements administered to subjects were changed, the results might have been different. However, it appears that adult males can adjust appropriately to variations in dietary Ca:P ratios from 1:1 to 1:3 at least for short periods of time.

We also were interested in determining the effects of a diet with a Ca:P ratio of 1:1 but with high levels of both calcium and phosphorus on mineral metabolism of human subjects for two reasons. 1) It is often assumed that the optimal Ca:P ratio for adults is 1:1 (21). 2) Several investigators have observed that the addition of high levels of both calcium and phosphorus to diets depressed utilization of iron (28,44,45) and zinc (41,47) by laboratory animals and humans much more than just the addition of high levels of phosphorus. However, in this study subjects retained as much magnesium, manganese and selenium when fed the HCaHP diet as when fed the other two diets. Similarly Leichsenring, et al. (26) and Snedeker, et al. (27) have observed that the addition of high levels of calcium and phosphorus to human diets did not depress significantly overall retention of iron (27), zinc (27), copper (27) and magnesium (26) by human subjects. In contrast, Kim (7) observed depressed retention of magnesium and Monsen and Cook (28) observed depressed retention of

iron among human subjects when both phosphorus and calcium supplements were administered. These differences in results among studies may reflect differences in the forms of phosphorus and calcium administered , i.e. calcium phosphate salts versus glycerol phosphate and calcium gluconate, or differences in the total composition of diets, i.e. ascorbic acid and phytate levels.

In summary, wide variations in calcium and phosphorus levels (800 to 2400 mg daily) and ratios (1:1 to 1:3) did not appear to affect utilization of magnesium, manganese and selenium by human subjects in this study. More subjects were able to achieve positive balance quickly with regard to phosphorus when the dietary level of phosphorus was raised above 800 mg phosphorus daily. However, the addition of high levels of calcium to the diet did not foster a significant improvement in overall retention of calcium by these subjects during a two week period.

REFERENCES

1. HULLEY, S.B., VOGEL, J.M., DONALDSON, C.L., BAYERS, J.H., FRIEDMAN, R.J. and ROSEN, S.N. The effect of supplemental oral phosphate on the bone mineral changes during prolonged bed rest. J. Clin. Invest., 50: 2506-2518, 1971.

2. MALM, O.J. On phosphates and phosphoric acid as dietary factors in the calcium balance of man. Scand. J. Clin. and Lab. Invest. 5: 75-84, 1953.

3. SPENCER, H., KRAMER, L., OSIS, D. and NORRIS, C. Effect of phosphorus on the absorption of calcium and on the calcium balance in man. J. Nutr. 108: 447-457, 1978.

4. SPENCER, H., MENCZEL, J., LEWIN, I. and SAMACHSON, J. Effect of high phosphorus intake on calcium and phosphorus metabolism in man. J. Nutr. 86: 125-132, 1965.

5. HEGSTED, M., SCHUETTE, S.A., ZEMEL, M.B. and LINKSWILER, H.M. Urinary calcium and calcium balance in young men as affected by level of protein and phosphorus intake. J. Nutr. 111: 553-562, 1981.

6. GOLDSMITH, R.S., JOWSEY, J., DUBE, W.J., RIGGS, B.L., ARNAUD, C.D. and KELLY, P.J. Effects of phosphorus supplementation serum parathyroid hormone and bone morphology in osteoporosis. J. Clin. Endocrinol. Metab. 43: 523-532, 1976.

7. KIM, Y. Effect of level of protein, calcium, phosphorus and magnesium metabolism in the young adult male. Ph. D. thesis, University of Wisconsin, Madison, 1977.

8. ZEMEL, M.B. AND LINKSWILER, H.M. Calcium metabolism in the young adult as affected by level and form of phosphorus intake andlevel of calcium intake. J. Nutr. 111: 315-324, 1981.

9. PATTON, M.B., WILSON, E.D., LEICHSENRING, J.M., NORRIS, L.M., and DIENHART, C.M. The relation of calcium to phosphorus ratio to the utilization of these minerals by eighteen young college women. J. Nutr. 50: 373-382, 1953.

10. LEICHSENRING, J.M., NORRIS, L.M., LAMISON, S.A., WILSON, E.D. and PATTON, M.B. The effect of level of intake on calcium and phosphorus metabolism in college women. J. Nutr. 45: 407-418, 1951.

11. ANDERSON, G.H., DRAPER, H.H. and KASTELIC, J. Enhanced bone resorption in adult rats fed a high phosphate diet. Fed. Proceed. 29: 566, 1970.

12. ANDERSON, G.H. and DRAPER, H.H. Effect of dietary phosphorus on calcium metabolism in intact and parathyroidectomized adult rats. J. Nutr. 102: 1123-1132, 1972.

13. DRAPER, H.H., SIE, T-L. and BERGAN, J.G. Osteoporosis in aging rats induced by high phosphorus diets. J. Nutr. 102: 1133-1142, 1972.

14. SIE, T-L., DRAPER, H.H. and BELL, R.R. Hypocalcemia, hyperpaprthyroidism and bone resorption in rats induced by dietary phosphate. J. Nutr. 104: 1195-1201, 1974.

15. LAFLAMME, G.H. and JOWSEY, J. Bone and soft tissue changes with oral phosphate supplements. J. Clin. Invest. 51: 2834-2840, 1972.

16. KRISHNARAO, G.V.G. and DRAPER, H.H. Influence of dietary phosphate on bone resorption in senescent mice. J. Nutr. 102: 1143-1146, 1972.

17. KROOK, L., BARRET, R.B., USI, K. and WOLKE, R.E. Nutritional secondary hyperparathyroidism in the cat. Cornell Vet. 53: 224-240, 1963.

18. KROOK, L. and LOWE, J.E. Nutritional secondary hyperparathyroidism in the horse; with a description of the normal equine parathyroid gland. Path Vet. I (Suppl.): 1-92, 1964.

19. ANDERSON, M.P., HUNT, R.D., GRIFFITHS, H.J., MCINTYRE, K.W. and ZIMMERMAN, R.E. Long term effect of low dietary calcium:phosphates ratio on the skeleton of Cebus albifrons monkeys. J. Nutr. 107: 834-839, 1977.

20. PARFITT, A.M. The actions of parathyroid hormone on bone: Relation to bone remodeling and turnover, calcium homeostasis and metabolic bone disease. Part I: Mechanisms of calcium transfer between blood and bone and their cellular basis: Morphological and kinetic approaches to bone turnover. Metabolism 25: 809-844, 1976.

21. FOOD AND NUTRITION BOARD. Recommended Dietary Allowances. 9th edition. National Academy of Science, Washington, D.C., 1980.

22. MORRIS, E.R. and O'DELL, B.L. Relationship of excess calcium and phosphorus to magnesium requirement and toxicity in guinea pigs. J. Nutr. 81: 175-181, 1963.

23. FORBES, R.M. Mineral utilization in the rat. I. Effects of varying dietary ratios of calcium, magnesium and phosphorus. J. Nutr. 80: 321-326, 1963.

24. BUNCE, G.E., CHIEMCHAISRI, Y. and PHILLIPS, P.H. The mineral requirements of the dog. IV. Effect of certain dietary and physiologic factors upon the magnesium deficiency syndrome. J. Nutr. 76: 23-29, 1962.

25. SEELIG, M. The requirement of magnesium by the normal adult. Am. J. Clin. Nutr. 14:342-390, 1964.

26. LEICHSENRING, J.M., NORRIS, L.M. and LAMISON, S.A. Magnesium metabolism in college women: observations on the effect of calcium and phosphorus intake levels. J. Nutr. 45: 477-485, 1951.

27. SNEDEKER, S.M., SMITH, S.A. and GREGER, J.L. Effect of dietary calcium and phosphorus levels on the utilization of iron, copper, and zinc by adult males. J. Nutr. (Accepted).

28. MONSEN, E.R. and COOK, J.D. Food iron absorption in human subjects. IV. The effects of calcium and phosphate salts on the absorption of nonheme iron. Am. J. Clin. Nutr. 29: 1142-1148, 1976.

29. PETERS, T., Jr., APT, L. and ROSS, J.F. Effect of phosphates upon iron absorption studied in normal human subjects and in an experimental model using dialysis. Gastroenterology 61: 315-322, 1971.

30. SPENCER, H., ASMUSSEN, C.R., HOLTZMAN, R.B. and KRAMER, L. Metabolic balances of cadmium, copper, manganese, and zinc in man. Am. J. Clin. Nutr. 32: 1867-1875, 1979.

31. PECOUD, A., DONZEL, P. and SCHELLING, J.L. Effect of foodstuffs on the absorption of zinc sulfate. Clin. Pharm. Therap. 17: 469-474, 1975.

32. WACHTEL, L.W., ELVEHJEM, C.A. and HART, E.B. Studies on the physiology of manganese in the rat. Am. J. Physiol. 140: 72-82, 1943.

33. WILGUS, H.S. JR. and PATTON, A.R. Factors affecting manganese utilization in the chick. J. Nutr. 18:35-45, 1939.

34. HAWKINS, G.E. JR., WISE, G.H., MATRONE, G., WAUGH, R.K. and LOTT, W.L. Manganese in the nutrition of young dairy cattle fed different levels of calcium and phoshporus. J. Dairy Sci. 38: 536-547, 1955.

35. GREGER, J.L. and MARCUS, R.E. Effect of dietary protein, phosphorus and sulfur amino acids on selenium metabolism of adult males. Ann. Nutr. Metab. 25: 97-108, 1981.

36. GREGER, J.L. and SNEDEKER, S.M. Effect of dietary protein and phosphorus levels on the utilization of zinc, copper and manganese by adult males. J. Nutr. 110: 2243-2253, 1980.

37. FISKE, C.H. and SUBBAROW, Y.T. The colorimetric determination of phosphorus. J. Biol. Chem. 66: 375-300, 1925.

38. OH, S.H. Relationship between selenium content of food, blood and milk and the problem of retained placenta in dairy cows. M.S. thesis, University of Wisconsin, Madison, 1972.

39. PETERS, J.H. The determination of creatinine and creatine in blood and urine with photoelectric colorimeter. J. Biol. Chem. 146: 179-186, 1942.

40. STEEL, R.G.D. and TORRIE, J.H. Principles and Procedures of Statistics. McGraw-Hill, New York, 1960.

41. DOMINGUEZ, J.H., GRAY, R.W. and LEMANN, J. JR. Dietary phosphate deprivation in women and men: Effects on mineral and acid balances, parathyroid hormone and the metabolism of 25OH vitamin D. J. Clin. Endocrinol. Metab. 43: 1056-1068, 1976.

42. SCIENCE AND EDUCATION ADMINISTRATION. Food and Nutrient Intakes of Individuals in 1 Day in the United States, Spring 1977. Preliminary Report No. 2. U.S. Department of Agriculture; Washington, D.C. 1980.

43. BELL, R.R., DRAPER, H.H., TZENG, D.Y.M., SHIN, H.K. and SCHMIDT, G.R. Physiological responses of human adults to foods containing phosphate additives. J. Nutr. 107: 42-50, 1977.

44. POND, W.G., WALKER, E.F. JR. and KIRTLAND, D. Effect of dietary Ca and P levels from 40 + 100 kg body weight on weight gain and bone and soft tissue mineral concentrations. J. An. Sci. 46: 686-691, 1978.

45. WADDELL, D.G. and SELL, J.L. Effects of dietary calcium and phosphorus on the utilization of dietary iron by the chick. Poultry Sci. 43: 1249-1257, 1964.

46. STEWART, A.K. and MAGEE, A.C. Effect of zinc toxicity on calcium, phosphorus and magnesium metabolism of young rats. J. Nutr. 82: 287-295, 1964.

47. CABELL, C.A. and EARLE, I.P. Additive effect of calcium and phosphorus on utilization of dietary zinc. J. An. Sci. 24: 800-804, 1965.

NUTRITION RESEARCH, Vol. 1, pp. 327-338, 1981
0271-5317/81/040327-12$02.00/0 Printed in the USA.
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SERUM TRACE MINERALS DURING YEAST FEEDING

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ABSTRACT

We have consistently noted cutaneous reactions in subjects ingesting large quantities of yeast. Since depression of plasma zinc has been noted in a number of cutaneous disorders we prospectively measured serum zinc and zinc-binding proteins in six adult male subjects on yeast protein as their sole source of protein for eight days. Serum zinc, albumin, alpha$_2$-macroglobulin, copper, iron and serum amyloid A (SAA) protein were measured before and at the end of the eight day interval. Five of the six individuals developed the characteristic erythematous, papular rash of the palmar surface of the hands. Mean serum zinc in the six subjects decreased from 117.0 to 99.0 µg/dl ($p<0.05$), and mean serum albumin decreased from 4.9 to 4.7 g/dl ($p<0.01$). There was no significant change in alpha$_2$-macroglobulin, serum minerals or SAA protein. This study indicates that high intakes of yeast can be added to the list of conditions which are accompanied by depression of circulating zinc.

Key Words: Yeast, zinc, copper, serum proteins.

INTRODUCTION

With increasing pressure on the world's agricultural and fishery resources from an expanding population, the search for new and abundant protein sources for human consumption has turned to yeast. Studies in experimental animals demonstrate yeast to be a good source of protein, comparable in quality to purified casein (1), and of higher quality with added methionine (2,3). In human trials, however, yeast feeding has not been without side effects. The relatively high purine content of yeast is associated with increased blood and urinary uric acid in individuals consuming yeast (4). However, ingesting RNA-reduced yeast does not cause elevations of uric acid (5,6). In addition, gastrointestinal symptoms have been observed when some types of yeast were consumed in amounts as low as 15 g per day (7,8). The most troublesome adverse reaction to the consumption of food yeast, however, has been the incidence of transient cutaneous eruptions (9,10).

The nature of the cutaneous eruption is incompletely understood. It may be similar to an "id" reaction, a localized skin eruption occuring at a site distant to a primary fungal infection. Such id reactions usually occur on the hands and feet (11). Id

reactions are considered to be a hypersensitivity to yeast or fungal products transmitted via the bloodstream (12). Although the exact pathophysiology of the reaction is not understood the eruption invariably clears spontaneously when the yeast infection is eradicated.

Plasma zinc depression has been reported in a number of cutaneous disorders including psoriasis, icthyosis, venous leg ulcers (13) and acne (14,15). It has not been reported in association with dermatoses of presumptive allergic or immunologic origin. In our experience with trials of feeding yeast preparations as supplements to the diet we have noted papular erythematous reactions of the palmar surface of the hands and the soles of the feet of 10-20% of the subjects (6). We therefore investigated the possibility that the dermatitis reactions to yeast consumption in human subjects might be associated with changes in trace mineral concentration in subjects who developed a skin eruption while consuming yeast protein as the sole source of nitrogen.

METHODS

Subjects

Six young male adults with no previous participation in yeast-feeding experiments were studied. The yeast feeding protocol was approved by the Committee on the Use of Humans as Experimental Subjects at the Massachusetts Institute of Technology. Each subject agreed to participate by signing a consent declaration after being informed of the nature, purpose and risks of the study. Participants were in good health as determined by physical examination and routine laboratory hematology and chemistry determinations. Medical, nursing and dietetic supervision was provided throughout the entire study. The age, initial weight and response of each subject to the yeast feeding is given in Table 1.

TABLE 1.
Subject's Clinical Data
(Study Period April 23-May 1)

SUBJECT	AGE (YEARS)	INITIAL WEIGHT (kg)	RESPONSE(1)
H.D.	21	61.3	Rash on April 29
P.S.	19	71.1	None
S.K.	23	69.9	Rash on May 4
G.J.(2)	21	73.8	Rash on April 26
F.B.(3)	23	58.8	Rash on April 26
P.R.	21	82.2	Rash on Arpil 30

(1) All reactors developed an erythematous, papular rash of the hands and/or feet which eventually desquamated spontaneously.
(2) Stopped study on April 29.
(3) Stopped study on April 28.

Experimental Design

The first day of the study was a nitrogen-free but calorie-adequate period. Starting on the second day, and continuing for the following seven days, subjects ingested a liquid formula (Table 2) containing yeast protein, which provided 0.35 g protein/kg body weight/day and 29 kcal/kg body weight/day. The yeast used in the formula was from the sub-family Saccharomycoideae and was 57.6% protein (wt/wt). It was grown on methanol by the Provesta Corporation, Bartlesville, OK. The liquid formula diet, which contained yeast protein as the sole source of protein, was taken three times a day, at 8 a.m., 12 noon and 5 p.m.

TABLE 2.
Composition of Experimental Formula Diet

Food	Amount (g)
Yeast	42.6
Polycose[1]	200.0
Corn oil[2]	115.0
NaCl[3]	2.0
$Ca_{10}(OH)_2(PO_4)_6$[4]	1.6
K_2HPO_4[5]	4.4
Avicel® [6]	5.0
Water	450.0
Total	820.6

(1) Polycose® (glucose polymer): Ross Laboratories, Columbus, OH. (2) Mazola Corn Oil: Best Foods, Englewood Cliffs, NJ. (3) Sodium Chloride Crystals: A.R. Mallinckrodt Inc., Paris, KY. (4) Calcium Phosphate Tri-basic Powder: J.T. Baker Chemical Co., Phillipsburg, NJ. (5) Potassium Phosphate Dibasic Powder: J.T. Baker Chemical Co. (6) Avicel® Microcrystalline Cellulose (RC-591): Donated by F.M.C. Corporation, Food & Pharmaceutical Products, Philadelphia, PA.

Additional calories to bring subjects up to their usual daily caloric intake were supplied by protein-free beverages. A vitamin-mineral supplement was provided as a capsule once daily (Table 3). The supplemental micronutrient intake conformed to the recommendations of the Food and Nutrition Board (16), including trace elements (Table 3). Subjects were asked to report untoward symptoms to the physician supervising the study.

TABLE 3.

Vitamin and Mineral Supplements

One A Day Vitamins Plus Minerals (Miles Laboratories, Elkhart, IN)
One tablet a day, each tablet contains:

Vitamin A (5000 I.U.); Vitamin E (15 I.U.); Vitamin C (60 mg); Folic Acid (0.4 mg); Thiamine (1.5 mg); Riboflavin (1.7 mg); Niacin (20.0 mg); Vitamin B_6 (2.0 mg); Vitamin B_{12} (6.0 µg); Vitamin D (400.0 I.U.); Pantothenic Acid (10.0 mg); Iron (18.0 mg); Calcium (100.0 mg); Phosphorous (100.0 mg); Iodine (150.0 µg); Magnesium (100.0 mg); Copper (2.0mg); Zinc (15.0 mg).

Trace Mineral Mix
One capsule a day, each capsule contains:

MgO (398.0 mg Mg); $CuCl_2 \cdot 2H_2O$ (2.0 mg Cu); $MnSO_4 \cdot H_2O$ (1.6 mg Mn); $Na_2MoO_4 \cdot 2H_2O$ (0.08 mg Mo); $Cr_2(SO_4)_3 \cdot 15H_2O$ (0.05 mg Cr); Na_2SeO_3 (0.1 mg Se); $AlKSO_4 \cdot 12H_2O$ (1.61 mg Al); KI (0.29 mg I).

Choline (Plus Products, Irvine, CA)
Two tablets a day, each tablet contains:

Choline bitartrate (250.0 mg).

Sodium Chloride (Enseals No. 27) (Eli Lilly and Co., Indianapolis, IN). Diet supplemented with tablets to reach a total sodium intake of 120 meq/day, each tablet contains:

NaCl (1.0 g NaCl).

Mineral Determinations

Fasting blood samples were drawn from an antecubital vein prior to and at the termination of the eight-day feeding period. Samples were obtained on the fifth and sixth day respectively from two subjects who stopped the study prior to the eight-day interval because of severe cutaneous reactions. Blood allowed to clot was centrifuged and serum was transferred by disposable Pasteur pipettes to minimal-contamination Vacutainer® tubes (Becton-Dickinson, No. 6527) for freezing and storage prior to analysis. To prevent zinc contamination, pipettes and all glassware were immersed for twenty-four hours in a solution of redistilled nitric acid and deionized water (1:1), washed four times with bidistilled water, reimmersed overnight in demineralized water, and finally dried in air on a polyethylene-lined plastic glassware basket.

Serum concentrations of trace minerals were determined by atomic absorption spectrophotometry (Perkin-Elmer, Model 370). Zinc and copper were measured after dilution of serum samples (1:5) in demineralized water (17). Iron was determined similarly, but after deproteination of serum by 20% trichloroacetic acid (TCA). Standard solutions of zinc were prepared with a 5% glycerol, and for iron with 10% TCA in order to adjust aspiration rates to those of the sample dilutions.

Serum calcium was determined on an autoanalyzer (Technicon Instrument Corporation, Tarrytown, NY) according to Technicon method No. SE4-C003FJ4, September 1974. Serum for sodium and potassium was analyzed on an IL443 flame photometer (Instrumentation Laboratory, Inc., Lexington, MA).

Protein Determinations

Serum samples were assayed for albumin on an autoanalyzer by the Bromcresol green method (Technicon method No. SE4-0030-FD4, April 1974). Serum concentrations of total α_2-globulin were quantified by serum protein electrophoresis (Bulletin PM 701, Millipore Corporation, Acton, MA). Serum amyloid A (SAA) protein was determined according to the method of Sipe et al (18) using its cross reactivity with tissue fibril protein in a solid-phase radioimmunoassay.

Data Analysis

The Student t-test for paired data was used to determine the level of probability of changes in serum constituents before and after the experimental treatment (19).

RESULTS

Five of six individuals experienced a cutaneous reaction in association with the yeast feeding. The onset of the skin eruption is detailed in Table 1. As can be seen, one subject did not report any skin eruption; in another subject the rash did not develop until three days following completion of the study. The rashes were typically a papular eruption of the feet, mildly pruritic, and desquamating within a week. An example of the palmar rash is shown in Figure 1.

FIGURE 1.

The characteristic skin eruption on the palmar surface of the hands of one of the subjects.

Among the biochemical determinations of nutrients, the striking feature is the statistically significant decline in mean zinc levels for the group as a whole from 117.0 to 99.0 µg/dl ($p<0.05$). This is in contrast to the five other circulating minerals, none of which showed any suggestion of a change in association with yeast consumption (Table 4).

TABLE 4.

SERUM MINERALS AND PROTEIN CONCENTRATION
AS AFFECTED BY YEAST CONSUMPTION

SUBJECTS	ZN (µg/dl)		CU (µg/dl)		FE (µg/dl)		NA (meq/L)		K (meq/L)		CA (mg/dl)	
	pre	post	pre	post	pre	post	pre	post	pre	post	pre	post
H.D.	102	90	84	90	136	130	143	142	4.5	4.0	9.8	10.0
P.S.	156	102	120	120	70	122	145	144	3.8	3.9	10.2	10.0
S.H.	102	90	90	84	52	70	145	142	4.0	4.0	9.8	9.6
G.J.	126	120	120	114	62	76	142	145	4.4	4.4	9.8	10.0
F.B.	114	90	108	102	114	110	144	142	3.4	3.8	10.0	9.6
P.R.	102	102	120	120	72	126	144	145	3.8	4.0	9.8	10.0
$\bar{X}$	117.0	99.0	107.0	105.0	84.0	104.0	143.8	143.3	4.0	4.0	9.9	9.9
S.D.	21.4	11.9	16.3	15.5	33.0	26.2	1.2	1.5	0.4	0.2	0.2	0.2
Paired t-test	$p<0.05$		n.s.		n.s.		n.s.		n.s.		n.s.	

The principal binding proteins of zinc, albumin and α_2-globulin, were measured (Figure 2). Albumin concentration declined significantly from 4.9 to 4.7 g/dl ($p<0.01$) but no significant correlation between albumin and zinc concentration was seen ($r=0.069$; $p<0.1$; $n=12$). The correlation between the change in zinc before and after the feeding period with the change in albumin was 0.666 ($n=6$). Although not statistically significant, the simultaneous fall in both zinc and albumin in almost all subjects (Figure 2) is suggestive of a true association.

Alpha$_2$-globulin increased from 0.59 to 0.67 g/dl which was not significant by paired t-test analysis. However, an overall significant correlation between the absolute concentration of α_2-globulin and serum zinc for all determinations before and after yeast feeding ($n=12$) was noted with a correlation coefficient of $r=0.564$ ($p<0.05$).

The acute phase reactant protein marker, serum amyloid A protein, failed to show any significant change in response to yeast feeding. The mean serum SAA concentration actually decreased from 3.7 to 2.5 µg/ml during the study ($p>0.05$). The correlation coefficient for the change in zinc and the change in SAA was not statistically significant ($r=0.461$; $n=6$).

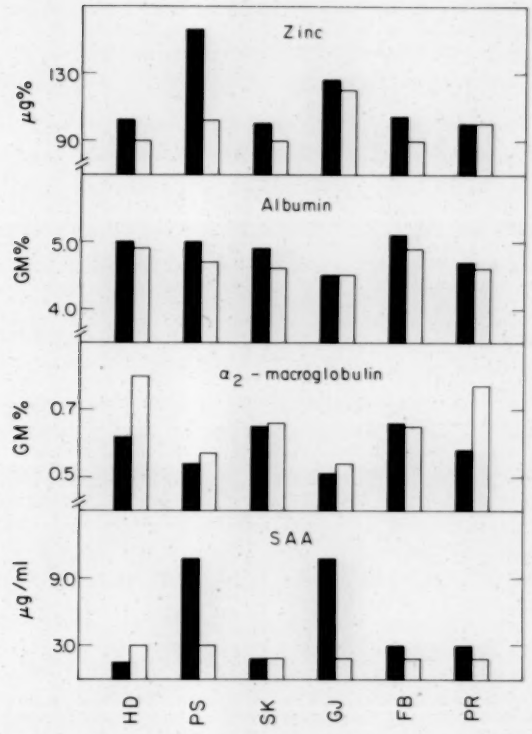

FIGURE 2.

The change in concentration of zinc, albumin, α_2-macroglobulin and SAA before (solid bars) and after (open bars) the eight-day feeding trial with yeast protein.

DISCUSSION

A unique and consistent decline in serum zinc concentration occurred as a result of feeding the experimental yeast protein as the sole source of nitrogen for the brief interval of eight days. Five of six subjects manifested a characteristic cutaneous reaction, but the depression of zinc levels also occurred in the individual who did not develop a rash. Circulating levels of two other trace minerals, copper and iron, and three macrominerals, calcium, sodium and potassium, were unchanged.

The pattern of behavior for zinc is reminiscent of the changes seen in infectious or inflammatory conditions such as bronchitis, pneumonia, cholecystitis and pelvic inflammatory disease (20,21), experimentally-induced infection with bacterial (22) or viral pathogens (23). This behavior is believed to be due to the action of a low-molecular weight polypeptide, leukocytic endogenous mediator(s) (LEM), produced by phagocytic white cells during stimulation by the pathogenic agent (24,25,26). Inflammatory stress

can also activate a LEM response as demonstrated by Solomons et al (27) in patients with Crohn's disease. A LEM response is also assiciated with an increase in acute-phase serum proteins (28,29, 30,31). In the present study, SAA, a protein with the biological characteristics of an acute-phase protein (32) was measured serially. SAA protein offered a unique potential as an acute-phase marker as its increase above usual circulating levels can be on the order of 100- to 200-fold (33,34). However no rise in SAA was detected. Also inconsistent with LEM-mediated redistribution of zinc is the fact that, in the clinical and experimental observations cited above, a parallel depression of circulating copper occurred. Our sampling interval might have missed any rise in copper, but iron depression should have occurred and been synchronous with the zinc decline if the mechanism was a classical LEM response (35).

Albumin is the dominant serum-binding protein for zinc (36), and albumin concentration or zinc-binding affinity can be a major determinant of zinc concentration (37). A slight decline in albumin concentration was observed during the feeding period, but the magnitude of the mean reduction in albumin was 4%, while that for zinc was 15.4%. As an explanation for the pattern of zinc change, therefore, the reduction of albumin concentration is not sufficient. The binding affinity of albumin is also potentially influenced by disease status; this possibility was not examined in this investigation.

A second protein, a component of the α_2-globulin fraction, accounts for 40% of zinc binding in serum (36). Not all protein of this class contains zinc, but apparently the total α_2-globulin fraction did not reflect the observed degree of zinc depression. In most situations of zinc depression during disease states, the α_2-globulin-associated zinc has remained unchanged, while the labile, loosely-bound fractions are consistently reduced (38).

The study did not allow for a comprehensive examination of zinc balance. Conceivably, serum zinc might have been reflecting a decreased entry or increased excretion of zinc from the body. It is unlikely, however, that zinc intake during the study period was lower than habitual as the diets contained at least 15 mg of zinc, the amount specified by the RDA (16). The exact intake of zinc by our subjects prior to the study is not precisely known, but recent surveys have demonstrated that spontaneous consumption of this mineral in self-selected American diets is well below the recommended allowance (39,40). The vitamin-mineral pills that provided the zinc were taken on an empty stomach away from mealtime, and thus, any reduction in the biological availability of zinc by factors in the yeast protein would not have influenced zinc absorption in this study. During the eight-day experimental period, therefore, the total consumption of zinc by the subjects presumably exceeded their customary intakes.

Another mechanistic consideration to explain the observed changes in zinc concentration would be the possibility of excessive wasting of endogenous zinc reserves. Increased loss of zinc into the intestine or into the urine could have been provoked in some way by the yeast-based diet. No data to include or exclude this mechanism are available.

Falchuck (38) in a prospective study of hospitalized patients reported that acute infirmities were associated with a transient fall in zinc. The present data indicate that the reaction of individuals to high intakes of an experimental yeast can be added to the list of acute conditions that are accompanied by depression

of circulating zinc. The actual expression of cutaneous signs does not appear to be prerequisite to the decline in zinc. Moreover, the zinc depression is not accompanied by changes in the concentration of other minerals, and it is not associated with the elevation of the one acute-phase reactant protein monitored. Changes in binding-protein concentrations cannot explain the disappearance of zinc from the circulation, and it is unlikely, but not impossible, that dietary zinc or patterns of zinc excretion during the eight-day period were factors in the decline of serum zinc. It has recently been suggested that low circulating zinc concentration, per se, with or without tissue depletion, may condition a number of pathophysiological responses in white cell function (41) and membrane stability (42). Further investigation into and characterization of the factors mediating zinc distribution in man merit concerted scientific attention.

ACKNOWLEDGEMENTS

This work was supported by a grant from the government of Egypt, the Provesta Co. (Bartlesville, OK), and an N.I.H. Research Training Fellowship (T-32-AM-070700). Dr. Solomons holds a Clinical Investigator Award from N.I.H. (1-K08-AM00715).

The authors gratefully acknowledge the assistance of Dr. K.P.W.J. McAdam in assaying the SAA. We thank Ms. Edwina Murray, the staff of the metabolic kitchen, the nursing personnel of the Clinical Research Center, and the volunteers whose willingness made this study possible.

REFERENCES

1. SURE, B. Dietary requirements for fertility and lactation; Brewers' and cultured food yeasts as sources of proteins and vitamin B complex for growth, reproduction and lactation. J . Am. Dietet. Assoc., 22:766-769, 1946.

2. GOYCO, J.A. and ASENJO, C.F. Net protein and growth promoting values of yeast prepared under identical conditions. J. Nutr., 38:517-526, 1949.

3. KLOSE, A.A. and FEVOLD, H.L. Nutritional value of yeast protein to the rat and the chick. J. Nutr., 29:421-430, 1945.

4. WASLIEN, C.I., CALLOWAY, D.H., MARGEN, S. and COSTA, F. Uric acid levels in men fed algae and yeast as protein sources. J. Food Sci., 35:294-298, 1970.

5. SCRIMSHAW, N.S. The future outlook for feeding the human race. In: Levures Cultives sur Alcanes-1972. H. Gounelle de Pontanel (ed). Centre de Recherches Foch, Paris, 1972, pp. 215-228.

6. SCRIMSHAW, N.S. and DILLON, J.C. Allergic responses to some single-cell proteins in human subjects. In: Single-Cell Protein Safety for Animals and Human Feeding. S. Garattini, S. Paglialunga and N.W. Scrimshaw (eds). Pergamon Press, Oxford, 1979, pp. 171-178.

7. GOYCO, J.A. Nitrogen balance of young adult consuming a deficient diet supplemented with Torula yeast and other nitrogenous products. J. Nutr., 69:49-57, 1959.

8. KLAPKA, M.R., DUBY, G.A. and PAVCEK, P.L. Torula yeast as a dietary supplement. J. Am. Dietet. Assoc., 34:1317-1320, 1958.

9. CALLOWAY, D.H. The place of SCP in man's diet. In: Single-Cell Protein. P. Davis (ed). Academic Press, London, 1974, pp. 129-146.

10. EDOZIEN, J.C. Yeast for human feeding - New data on safety. In: The PAG Compendium. World Mark International Documentation, New York, 1969, Protein Advisory Group Document 2.23/1, pp. C2083-C2092.

11. ODDS, F.C. Candida and Candidosis, University Park Press, Baltimore, MD, 1979, pp. 117-118.

12. HUMPHREY, J.S. and WHITE, R.G. Immunology for Students of Medicine. F.A. Davis, Co., Philadelphia, PA, 1963, pp. 327-328.

13. GREAVES, M. and BOYDE, T.R.C. Plasma zinc concentrations in patients with psoriasis, other dermatoses and venous leg ulceration. Lancet, 2:1019-1020, 1967.

14. MICHAELSSON, G., JUHLIN, L. and VAHLQUIST, A. Effects of oral zinc and vitamin A in acne. Arch. Dermatol., 113:31-36, 1977.

15. MICHAELSSON, G., VAHLQUIST, A. and JUHLIN, L. Serum zinc and retinol-binding protein in acne. Br. J. Dermatol., 96:283-286, 1977.

16. National Academy of Science Bulletin. Food and Nutrition Board, 9th edition. National Academy of Science, Washington, D.C., 1980.

17. KURZ, D., ROACH, J. and EYRING E.J. Direct determination of serum zinc and copper by atomic absorption spectrophotometry. Biochem. Med., 6:274-281, 1972.

18. SIPE, J.D., IGNACZAK, T.F., POLLOCK, P.S. and GLENNER, G.G. Amyloid fibril protein AA: Purification and properties of the antigenically-related serum component as determined by solid phase radioimmunoassay. J. Immunol., 116:1151-1156, 1976.

19. SNEDECOR, G.W. and COCHRAN, W.G. Statistical Methods. 6th edition. Iowa State University Press, Ames, Iowa, 1967.

20. SINHA, S.N. and GABRIELI, E.R. Serum copper and zinc in various pathological conditions. Am. J. Clin. Pathol., 54:570-577, 1970.

21. SULLIVAN, J.F., BLOTCKY, A.J., JETTON, M.M., HAHN, H.K.J. and BURCH, R.E. Serum levels of selenium, calcium, copper, magnesium, manganese and zinc in various human diseases. J. Nutr., 109:1432-1437, 1979.

22. WANNEMACHER, Jr., R.W., DuPONT, H.L., PEKAREK, R.S., POWANDA, M.C., SCHWARTZ, A., HORNICK, R.B. and BEISEL, W.R. An endogenous mediator of depression of amino acids and trace metals in serum during typhoid fever. J. Infect. Dis., 126:77-86, 1972.

23. PEKAREK, R.S., BURGHEN, G.A., BARTELLONI, P.J., CALIA, F.M. and BEISEL, W.R. The effect of live attenuated Venezuelan equine encephalomyelitis virus vaccine on serum iron, zinc and copper concentrations in man. J. Lab. Clin. Med., 76:293-303, 1970.

24. KAMPSCHMIDT, R.F. and UPCHURCH, H.F. The effect of endogenous pyrogen on plasma zinc concentration of the rat. Proc. Soc. Exp. Biol. Med., 134:1150-1152, 1970.

25. PEKAREK, R.S. and BEISEL, W.R. Characterization of the endogenous mediator(s) of serum zinc and iron depression during infection and other stresses. Proc. Soc. Exp. Biol. Med., 138:728-732, 1971.

26. PEKAREK, R.S., WANNEMACHER, Jr., R.W., CHAPPLE, F.E., POWANDA, M.C. and BEISEL, W.R. Further characterization and species specificity of leukocytic endogenous mediator (LEM). Proc. Soc. Exp. Biol. Med., 141:643-648, 1972.

27. SOLOMONS, N.W., ELSON, C.O., PEKAREK, R.S., JACOB, R.A., SANDSTEAD, H.H. and ROSENBERG, I.H. Leukocytic endogenous mediator in Crohn's disease. Infect. Immunol., 22:637-639, 1978.

28. EDDINGTON, C.L., UPCHURCH, H.F. and KAMPSCHMIDT, R.F. Effect of extracts from rabbit leukocytes on levels of acute phase globulins in rat serum. Proc. Soc. Exp. Biol. Med., 136:159-164, 1971.

29. PEKAREK, R.S., POWANDA, M.C. and WANNEMACHER, Jr., R.W. The effect of leukocytic endogenous mediator (LEM) on serum copper and ceruloplasmin concentration in the rat. Proc. Soc. Exp. Biol. Med., 141:1029-1031, 1972.

30. PEKAREK, R.S., WANNEMACHER, Jr., R.W., POWANDA, M., ABELES, F., MOSHER, D., DINTERMAN, R. and BEISEL, W. Further evidence that leukocytic endogenous mediator (LEM) is not endotoxin. Life Sci., 14:1765-1776, 1974.

31. WANNEMACHER, Jr., R.W., PEKAREK, R.S., THOMPSON, W.L., CURNOW, R.T., BEALL, F.H., ZENSER, T.V., DeRUBERTIS, F.R. and BEISEL, W.R. A protein from polymorphonuclear leukocytes (LEM) which affects the rate of hepatic amino acid transport and synthesis of acute-phase globulins. Endocrinology, 96:651-661, 1975.

32. McADAM, K.P.W.J. and SIPE, J.P. Murine model for human secondary amyloidosis: genetic variability of the acute-phase serum protein SAA response to endotoxins and casein. J. Exp. Med., 144:1121-1127, 1976.

33. McADAM, K.P.W.J., ELIN, R.J., SIPE, J.P. and WOLFF, S.M. Changes in human serum amyloid A and C-reactive protein after etiocholanolone-induced inflammation. J. Clin. Invest., 61:390-394, 1978.

34. McADAM, K.P.W.J. and RYAN, J.L. C57BL/10CR mice: Nonresponders to activation by the lipid A moiety of bacterial lipopoly-saccharide. J. Immunol., 120:249-253, 1978.

35. PEKAREK, R.S., BOSTIAN, K.A., BARTELLONI, P.J., CALIA, F.M. and BEISEL, W.R. The effect of Francisella tularensis infection on iron metabolism in man. Amer. J. Med. Sci., 258:14-25, 1969.

36. PARISI, A.F. and VALLEE, B.L. Isolation of a zinc α_2-macroglobulin from human serum. Biochemistry, 9:2421-2426, 1970.

37. SOLOMONS, N.W. On the assessment of zinc and copper nutriture in man. Am. J. Clin. Nutr., 32:856-871, 1979.

38. FALCHUK, K.H. The effect of acute disease and ACTH on serum zinc proteins. NEJM, 296:1129-1137, 1977.

39. HOLDEN, J.M., WOLF, W.R. and MERTZ, W. Zinc and copper in self-selected diets. J. Am. Dietet. Assoc., 75:23-29, 1979.

40. KLEVAY, L.M., RECK, S.J. and BARCOME, D.F. Evidence of dietary copper and zinc deficiencies. JAMA., 24:1916-1918, 1979.

41. WESTON,W.L., HUFF, J.C., HUMBERT, J.R., HAMBRIDGE, K.M. NELDNER, K.H. and WALRAVENS, P.A. Zinc correction of defective chemotaxis in acrodermatitis enteropathica. Arch. Dermatol., 113:422-425, 1977.

42. BETTEGER, W.J., FERNANDEZ, M.S. and O'DELL, B.L. Effect of zinc deficiency on the zinc content of rat red cell membranes (Abstr.). Fed. Proc., 39:896, 1980.

NUTRITION RESEARCH, Vol. 1, pp. 339-347, 1981
0271-5317/81/040339-09$02.00/0 Printed in the USA.
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LIPID UTILIZATION OF HUMAN ADULTS AS AFFECTED BY DIETARY BRAN SUPPLEMENTATION

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ABSTRACT

Twenty-one adult men and women participated in two studies to compare the effects of selected brans on the utilization of dietary fat. Fecal fat excretion was measured as well as total weight of feces. Blood serum cholesterol, triglycerides, and phospholipids were determined. Results indicated that fecal fat excretion tended to increase with bran supplementation of diets. Wheat bran had a greater effect than did either rice or corn bran. No statistically significant changes in blood lipids were associated with the various types of fiber. However, serum lipids of subjects were substantially lower when subjects received the experimental diets than when they were consuming their self-selected diets prior to the start of the study. The experimental diet was moderately low in fat and very low in cholesterol. The effectiveness of added bran in influencing lipid utilization may vary with the kinds of bran employed and also with the total amount of dietary fat or other dietary parameters.

Lipids Corn bran Rice bran Cholesterol Triglycerides

INTRODUCTION

Coronary heart disease (CHD) is the major cause of death in the United States and other developed countries (1). Several risk factors have been identified to predict individuals who have an increased chance of having CHD. Some of these, such as genetics and age, are not changeable. One risk factor which can be altered, however, is elevated blood serum lipid levels (2).

An increase in dietary fiber has been suggested as one method by which this can be accomplished (3,4,5). The mechanism by which fiber may do this is not clear but has been postulated to be related to the binding properties of different fiber components (6). Since brans vary in their fiber make-up and fibers vary in their chemical and physical properties, variation in physical/chemical influences of bran can be expected. Therefore, in research with dietary fiber, the need for specificity of type of bran and physiological effects of each type needs to be investigated. This is particularly true with regard to effects of dietary fiber on lipid utilization and circulating blood lipids. The objective of the current research was to study the

effect of wheat bran, corn bran, and rice bran on lipid utilization and the serum cholesterol, triglyceride, and phospholipid levels in human adults under controlled conditions.

EXPERIMENTAL PROCEDURE

Experimental Plan

The project consisted of two 34-day studies involving 21 subjects. Each study was composed of a 3-day pre-period, a 7-day adjustment period, three 7-day randomized experimental periods, and a 3-day post-period (Table 1).

TABLE 1

Experimental Plan for Studies A and B

Period[1]	No. of Days	Diet
Group A		
Self-selected	3	---
Adjustment	7	No fiber supplementation
Experiment 1	7	Corn bran supplementation[2]
Experiment 2	7	Wheat bran supplementation[3]
Experiment 3	7	No fiber supplementation
Self-selected	3	---
Group B		
Self-selected	3	---
Adjustment	7	No fiber supplementation
Experiment 1	7	Rice bran supplementation[4]
Experiment 2	7	Wheat bran supplementation
Experiment 3	7	No fiber supplementation
Self-selected	3	---

[1] Experimental periods were randomly arranged for subjects.
[2] Corn bran supplement consisted of 50.74% neutral detergent fiber (13).
[3] Wheat bran supplement consisted of 42.06% neutral detergent fiber (13).
[4] Rice bran supplement consisted of 23.30% neutral detergent fiber (13).

The purpose of the pre- and post-study periods was to gain some information on normal blood serum lipids and stool fat content with usually consumed self-selected diets. The adjustment period allowed subjects time to adapt to the basal diet (Table 2) used in the experiment and calorie intake necessary for weight maintenance was determined during this time. The laboratory-controlled diet which was fed each day provided moderately low fat and low amounts of cholesterol. Different amounts of jelly, soft drinks and hard candies were used to provide extra calories. No fiber was added in the adjustment period and during one experimental period of each study. During the other two experimental periods, fiber in the form of bran was incorporated into the bread in the daily diet: 20 g of wheat bran and 20 g of corn bran were added in Study A and 20 g of wheat bran and 20 g of rice bran were added in Study B. Subjects collected all excreta. Fasting blood samples were drawn from subjects on the last day of each experimental period.

TABLE 2

Basal Diet

Item	Amount/subject/day
Orange juice	100 g
Applesauce (sweetened)	100 g
Peaches	100 g
Pears	100 g
Green beans	50 g
Tomatoes	50 g
Corn flakes	21 g
Rice, converted	50 g
Peanut butter	25 g
Nonfat dry milk	99 g
Mashed potatoes	25 g
Bread	varied[1,2]
Cole slaw	100 g
Ground beef	50 g[3]
TVP	50 g[3]
Soft drinks	varied[1]
Hard candy	varied[1]
Jelly/honey	varied[1]
Margarine	varied[1]

[1]Fed in amounts to meet caloric needs for weight maintenance of individuals. Amounts were kept constant for each individual.
[2]Bread consisted of yeast, corn oil, sugar, salt, white enriched flour and water.
[3]TVP was given instead of ground beef to subjects who were vegetarians (subjects 2308, 2309, 2317, 2318 and 2319).

Subjects

Eleven adults (2 men and 9 women) in Study A and ten adults (all women) in Study B served as subjects. The 21 subjects ranged in age from 20 to 50 years with a mean of 25 years, in weight from 29.5 to 93.6 Kg with a mean of 59.2 Kg, and in height from 132 to 175 cm with a mean of 164 cm. By ethnic/race classification, all were white/Americans except one who was Chinese. Five of the subjects were vegetarians and 16 were omnivores. Modest alterations were made in the usual basal diet as shown in Table 2 to accomodate the vegetarian subjects. Among the checks used in this laboratory for ascertaining adherence to the total program were the following: measurement of 24-hour urinary nitrogen excretions, observation of subjects while consuming meals, and close-checking of meal trays. Subjects received clearance from the University of Nebraska Health Services on the basis of health records. This project was reviewed and approved by the University of Nebraska Institutional Committee on Investigation Involving Human Subjects.

Laboratory Analysis

Subjects collected all feces. Collections were composited into 5-day lots for both studies. Carmine (red) marker was given on the first day of the period. Brilliant blue marker and glass beads were given on the third day. Feces from the red marker to the beginning of the blue marker were discarded. Composites included all fecal matter from the beginning of the blue

marker to the beginning of the red marker. Fecal fat was determined by an A.O.A.C. modification of the Goldfisch method of fat extraction (7). Fasting venous blood samples were drawn from each subject at the beginning of the study and at the end of each experimental period by the Pathological Laboratories of the University of Nebraska Student Health Services. Blood serum was analyzed for phospholipids by a modification of the methods of Bloor (8), Boumann (9) and Fiske and Subbarow (10). Blood serum levels of cholesterol and triglycerides were done by Stat Laboratories of Kansas.

Statistical Analysis

Resulting data from the experiment outlined above were subjected to statistical analysis including Duncan's New Multiple Range Test with consultation from the University of Nebraska Institution of Agriculture and Natural Resources' Statistical Laboratories.

RESULTS AND DISCUSSION

Mean total fecal fat excretions, total dry fecal weights, total wet fecal weights, fecal fat excretions per gram dry feces and per gram wet feces are given in Table 3. Pre-period values in all cases are values of individuals while consuming self-selected diets; hence, they present "normal" or "usual" values for these individuals.

Total fecal fat excretions of subjects while in the pre-study, no added fiber, added wheat bran or added corn bran in Study A were 13.41, 5.74, 8.78, and 6.07 g per period, respectively. All values are expressed in terms of excretions for 5 days. The pre-period was three days in length; hence, values were proprotionally increased for comparison purposes adjusted by ratio from 3 to 5 days. During the pre-study highest fecal fat excretions were found. They were statistically different from all values during experimental periods. Added wheat bran resulted in significantly higher fecal fat excretions in comparison to values from both the no added fiber and the added corn bran periods. The high fat excretion on the pre-study could be due to a high fat diet that is found in the typical American diet. The basal diet used on this study contained approximately 30% of calories from fat, an ideal for Americans rather than what is typical. Since diets during all experimental periods were of the same fat content, the higher fat excretion with the added wheat bran can be assumed to be the result of the added fiber or other constituents from the wheat bran. However, the added corn bran fiber seemingly had no effect on fat absorption.

In Study B, total fat excretion for the pre-study, no added fiber period, added wheat bran period, and added rice bran period were 23.30, 4.77, 8.36, and 6.69 g per period, respectively. As in the first study, subjects showed high fat excretion during the free-choice diet period which was statistically different from the experimental periods. Of the experimental period values, the added wheat bran resulted in the highest level and the no added fiber period had the lowest fat excretion. Rice resulted in an intermediate trend. However, none of these trends was statistically significant.

Fecal fat excretion per gram wet feces were 0.030, 0.014, 0.015, and 0.014 in Study A and 0.050, 0.022, 0.022, and 0.021 in Study B for pre-study, no added fiber, added wheat bran and either added corn bran (Study A) or rice bran (Study B) periods, respectively. Both studies indicated a greater amount of fat per gram of wet feces in the pre-study. This could be expected with total fecal fat in this period being highest. It is interesting to note that

TABLE 3

Mean Total Fecal Fat Excretion, Total Wet Feces, Total Dry Feces, Fecal Fat Excretion per gram Wet Feces, and Fecal Fat Excretion per gram Dry Feces--Studies A and B[1]

Diet Code[2]	Total Fecal Fat g/period[3]	Total Wet Feces g/period[3]	Total Dry Feces g/period[3]	Fecal Fat Excretion per gram Wet Feces[3]	Fecal Fat Excretion per gram Dry Feces[3]
Study A					
Pre	13.41[a] ±4.73	592[a] ±281	130[c] ±47	0.030[a] ±.012	0.106[a] ±.029
NF	5.74[c] ±1.05	494[b] ±189	111[d] ±23	0.014[b] ±.006	0.054[b,c] ±.062
+W	8.78[b] ±2.52	607[a] ±180	144[a] ±31	0.015[b] ±.004	0.062[b] ±.012
+C	6.07[c] ±1.53	480[b] ±162	135[b] ±29	0.014[b] ±.004	0.047[c] ±.014
Study B					
Pre	23.30[a] ±8.39	487[a] ±147	152[a] ±40	0.050[a] ±.020	0.156[a] ±.052
NF	4.77[b] ±1.46	270[d] ±136	71[d] ±28	0.022[b] ±.008	0.073[b] ±.014
+W	8.36[b] ±2.25	400[b] ±90	112[b] ±19	0.022[b] ±.006	0.075[b] ±.013
+R	6.69[b] ±1.57	344[c] ±116	102[c] ±27	0.021[b] ±.006	0.067[b] ±.013

[1]Different letter superscripts indicate values significantly different from one another at $P<0.05$ (analysis of variance and Duncan's New Multiple Range Test).

[2]Diet Code:

NF = Basal diet with no fiber supplementation
+C = Basal diet + 20 g corn bran
+W = Basal diet + 20 g wheat bran
+R = Basal diet + 20 g rice bran

[3]Periods represent 5-day fecal composites. Pre-period values adjusted by ratio from 3 to 5 days.

the values were almost identical in each respective study for fecal fat excretion concentration on a gram wet fece basis during the experimental periods in spite of variation in total fecal fat excretion. This indicates that total fece production rather than fat absorption might be the mechanism of importance.

Mean fecal fat excretion per gram dry feces were 0.106, 0.054, 0.062, and 0.047 in Study A for pre-study, no added fiber, added wheat bran, or added corn bran, respectively. The pre-study feces contained almost double the fat concentration of feces for the experimental periods and these values were statistically different from those of the experimental periods. Added wheat and corn bran resulted in significantly different amounts of fecal fat (dry weight basis) but neither was different from the no added fiber period. This again stresses the importance of total stool production although seemingly wheat bran showed some trends toward decreased fat absorption. In Study B the pre-study mean value of fecal fat per gram dry feces was 0.156, twice the amount of fat per gram of dry feces of the experimental periods. The mean values for no added fiber, added wheat bran, and added rice bran were 0.073, 0.075, and 0.067, respectively. These values were not signifcantly different from one another.

Total wet feces of subjects while on pre-study, no added fiber, added wheat bran and added corn bran were 592, 474, 607, and 480 grams per period, respectively, on Study A. The addition of wheat bran produced the most wet feces, and although these levels were not statistically different from the pre-study, this value was statistically different from those of the no added fiber or the added corn bran periods. Fecal production during the no added fiber period and the added corn period did not differ significantly. Study B had values of 487, 270, 400, and 344 g for the pre-study, no added fiber, added wheat bran and added rice bran periods, respectively. During the pre-study period, the highest volume of wet feces was produced, a level which was statistically different from that of the added wheat bran period during which the second highest volume was produced. The added wheat bran period values differed significantly from the no added fiber period and the added rice bran period. The added rice bran period value was significantly different from the no added fiber period value which had the lowest wet feces total.

Mean dry fece production values were statistically different in both studies. Study A had means of 130, 111, 144, and 135 g per period, while Study B had means of 152, 71, 112, and 102 g per period for the pre-study, no added fiber, added wheat bran and added corn bran (Study A) or added rice bran (Study B) periods, respectively. The pre-study value is second lowest in Study A and highest in Study B. Among the three experimental periods, the added wheat bran period had the highest value in both studies. The second value is either the added corn bran period or added rice bran period, in their respective studies. The no added fiber period had the lowest value.

Mean serum cholesterol, triglycerides, and phospholipids of subjects while receiving the various experimental diets are given in Table 4.

Mean serum cholesterol values were 172, 147, 152, and 147 for Study A and 165, 143, 142, and 142 mg per dl in Study B for pre-study, no added fiber, added wheat bran, and either added corn bran in Study A periods or added rice bran in Study B periods, respectively. Serum cholesterol declined significantly with all controlled feeding periods in comparison to the pre-self-selected diet period. This may have been due to the lower total fat and total cholesterol content of the basal diet in comparison to the typical American diet these subjects probably consumed. Since more fat was excreted with the wheat bran, it might be expected to result in a greater drop in

TABLE 4

Mean Serum Cholesterol, Triglyceride and Phospholipid Values According to Diets--Studies A and B[1]

Diet Code[2]	Serum Cholesterol mg/dl	Serum Triglyceride mg/dl	Serum Phospholipid mg/dl
Study A			
Pre	172[a]±19	124[a]±71	223[a]±30
NF	147[b]±20	99[a]±46	204[a]±37
+W	152[b]±19	118[a]±61	218[a]±23
+C	147[b]±21	114[a]±58	215[a]±45
Study B			
Pre	165[a]±28	78[a]±26	218[a]±30
NF	143[b]±23	74[a]±26	192[b]±35
+W	142[b]±24	84[a]±33	181[b]±28
+R	142[b]±22	78[a]±20	188[b]±36

[1]Different letter superscripts indicate values significantly different from one another at $P<0.05$ (analysis of variance and Duncan's New Multiple Range Test).

[2]Diet Code:

NF = Basal diet with no fiber supplementation
+C = Basal diet + 20 g corn bran
+W = Basal diet + 20 g wheat bran
+R = Basal diet + 20 g rice bran

serum cholesterol than would other diets. Actually, this was not demonstrated in the current project. Since basal diets resulted in low values for serum cholesterol, perhaps the physiological limits were reached for this parameter. In two studies by Kies et al. (11), serum cholesterol mainly declined with the increased feeding of bran. Mean cholesterol levels of 196, 194, and 182 were reported when 0, 5 or 10 g wheat bran were fed, and 172, 174, and 150 mg per dl cholesterol levels were reported when 0, 10 or 20 g wheat gran were added to the basal diets. The same wheat bran and same basal diet were used in the second study of Kies et al. as were used in the current project so the discrepancy in results are difficult to explain.

Serum triglyceride levels showed no statistical differences among means of 124, 99, 118, and 114 in Study A and 78, 74, 84 and 78 mg per dl in Study

B for pre-study, no added fiber, added wheat bran, and added corn bran (Study A) periods or added rice bran (Study B) periods, respectively. Trends toward increases with feeding bran as compared to no added fiber were observed. Very often factors resulting in declines in blood serum cholesterol levels result in increases in blood serum triglyceride levels. Although no significant changes in either parameter were demonstrated, these trends may be of physiological significance. Kies et al. (11) also reported no significant changes in blood serum triglyceride levels as a result of wheat bran or fiber supplementation of diets.

Serum phospholipid values were 223, 204, 218, and 215 in Study A and in Study B were 218, 192, 181, and 188 mg per dl for the pre-study, no added fiber, added wheat bran and added corn bran in Study A periods or added rice bran in Study B periods, respectively. In Study A no significant differences were found but in Study B a lowering of phospholipids with the three controlled feeding periods was seen in comparison to the pre-study value. In general, phospholipid levels are more resistant to change than are triglyceride or cholesterol levels. Usually, phospholipid levels tend to reflect cholesterol levels; that is if cholesterol levels increase so do phospholipid levels and if cholesterol levels decrease, so do phospholipid levels. For this reason and also because of technical difficulties in analyses, phospholipid levels are seldom reported in research literature. In this study, in general, a decline in cholesterol levels from that observed in self-selected diets was accompanied by a decline in phospholipid levels even though these changes were not always statistically significant. However, fiber supplementation itself had no effect.

In conclusion, wheat bran was shown to have a greater effect on fecal fat excretion that was corn bran when expressed on the basis of total fecal fat or fat per gram dry feces. Rice bran had a directionally similar effect to that of wheat bran. Lo, (12) in studying the zinc and copper balances with the same studies, also found that wheat bran and rice bran had greater effects that the corn bran on the utilization of these minerals. No significant effects of bran on cholesterol, phospholipid or triglyceride levels were demonstrated. In this study it appears that a lowered intake of dietary fat and/or dietary cholesterol may mask any additional effects on circulating blood lipids which might be the result of added dietary fiber.

SUMMARY

The objective of the current research was to investigate the effects of wheat bran, corn bran and rice bran on lipid absorption and serum lipid levels. The project consisted of two 34-day studies involving twenty-one subjects. Each study was composed of a 3-day pre-period, a 7-day adjustment period, three 7-day randomized experimental periods, and a 3-day post-period. During the experimental periods a basal diet plus no added fiber, 20 g added wheat bran and 20 g added corn bran were used in Study A. In Study B, similar diets were used except that 20 g added rice bran, rather than 20 g corn bran were added in one period. Fecal fat excretion was measured as well as total weight of feces. Serum cholesterol, triglycerides, and phospholipids were determined.

Results showed total fecal fat excreted differed significantly for the pre-study and experimental periods. In genral, wheat bran supplementation resulted in higher fecal fat excretion in comparison to excretions when no bran supplements were used or when corn bran supplements were employed. Rice bran effects were directionally similar to those of wheat bran but of lesser magnitude. While an increase in stool volume appears to accompany the added wheat fiber, the wheat fiber also apparently decreases fat absorption since there is increased fat in the stools when dry weights of stools are used. The other fibers studied have a lesser effect on fat absorption when fat per gram dry weight of stool is considered.

Serum cholesterol declined significantly from the pre-study values to experimental diet levels. The lowered values did not show significant differences between added fiber periods and the no added fiber period. Serum triglycerides showed no significant differences. Serum phospholipids did not change in Study A but in Study B significantly decreased from the pre-study.

REFERENCES

1. AMERICAN HEART ASSOCIATION. Heart Facts. 1975, p. 9.

2. _____. Diet and coronary heart disease. Joint policy statement of the American Medical Association Council on Foods and Nutrition and the Food and Nutrition Board of the National Academy of Sciences-National Research Council. J.A.M.A. 222:13, 1972.

3. BURKITT, D.P. AND TROWELL, H.C. Refined Carbohydrate Foods and Diseases. Academic Press, Inc., London, 1975.

4. CARLSON, L.A. AND BOTTIGER, L.E. Ischaemic heart disease in relation to fasting values of plasma triglycerides and cholesterol: Stockholm Prospective Study. Lancet 1:865, 1972.

5. BURKITT, D.P., WALKER, A.R.P., AND PAINTER, N.S. Dietary fiber and disease. J.A.M.A. 220:1068, 1974.

6. EASTWOOD, M.A., AND MITCHELL, W.D. Physical properties of fiber: a biological evaluation in Fiber in Human Nutrition. G.A. Spiller and R.J. Amen (ed.). Plenum Press, New York, 1976.

7. AMERICAN ASSOCIATION OF CEREAL CHEMISTS. Approved methods of A.A.C.C., St. Paul, Minnesota, 44-01, 1969.

8. BLOOR, W.R. A method for the determination of fat in small amounts of blood. J. Biol. Chem. 17:377, 1914.

9. BOUMANN, E.J. On estimation of organic phosphorus. J. Biol. Chem. 59: 667, 1924.

10. FISKE, C.H. AND SUBBAROW, Y. The colormetric determination of phosphorus. J. Biol. Chem. 66:375, 1925.

11. KIES, C., FOX, H.M. AND VAUGHAN, L. Wheat bran supplementary effects on nutritional status of human adults. 6th International Cereal and Bread Congress AACC Meeting. Winnipeg, Canada, Sept., 1978.

12. LO, B. The effect of selected brans (wheat, corn and rice) on zinc and copper utilization in humans. Unpublished M.S. thesis, University of Nebraska-Lincoln, 1979.

13. WESTRING, M.E. Fecal fiber excretion of humans as affected by selected bran intake. Unpublished M.S. thesis, University of Nebraska- Lincoln, 1979.

Funded by Nebraska Agriculture Experiment Station Project 91-007 and U.S.D.A. S.E.A. Project NC-95. Published as Nebraska Agriculture Experiment Station Journal article series number 5638.

NUTRITION RESEARCH, Vol. 1, pp. 349-361, 1981
0271-5317/81/040349-13$02.00/0 Printed in the USA.
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THE ROLE OF COBALAMIN IN SYNTHESIS OF PROTEIN AND IMMUNOGLOBULINS BY HUMAN PERIPHERAL LYMPHOCYTES

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ABSTRACT

The effects of vitamin B_{12} (cobalamin, Cbl) on IgG synthesis were measured in human peripheral blood lymphocytes (PBL) cultured for 6 days in the presence of pokeweed mitogen. The synthesis and release of total protein and IgG were measured by precipitation of the proteins in the media that had incorporated ^{3}H leucine. Cells from 5 Cbl deficient donors synthesized and released less protein and IgG than cells from healthy donors. Large amounts of free Cbl added in vitro enhanced the synthetic capacities of the Cbl deficient cells but not the normal cells. Cells from 6 healthy donors were somewhat depleted of Cbl in vitro by culture in the absence of any source of Cbl. When Cbl was added there was a significant enhancement of protein synthesis, but the increase in IgG synthesis, while considerable, did not reach significance. No effect of deficiency of Cbl or the addition of Cbl was observed on cell replication or blastogenesis. Apparently Cbl has an effect on protein and IgG synthesis and release by human PBL in addition to any potential effect on DNA synthesis and cell division.

Key Words: Cobalamin, Lymphocyte, Protein Synthesis, IgG Synthesis

INTRODUCTION

The performance of the present study was stimulated by a report of impaired immunoglobulin (Ig) production in an infant congenitally deficient in transcobalamin II (TC II) (1). Circulating levels of Ig did not develop in the child as expected and there was no antibody response to two common antigens. However, with restoration of his deficiency of cellular cobalamin (Cbl) by pharmacologic amounts of Cbl, the IgG levels became normal and remained so (1,2). Moreover, with therapy he abruptly developed antibodies against the antigens injected several months earlier even in the absence of a new challenge. Recently limited information from a second, similar patient has been reported (2).

The few formal animal studies of the function of Cbl in the production of Ig, reviewed recently by Newberne (3), do not relate closely to the present problem. Human studies have consisted chiefly of the measurement of serum Ig in adult forms of Cbl deficiency. Low levels, increasing after treatment with Cbl, have been reported in some series (4), but not in others (5,6). Of course, one must distinguish between low levels of Ig that might be observed

in the coincidence of pernicious anemia (PA) in a person with a primary Ig deficiency. Probably there are some important differences between the cellular Cbl defects of PA and those of the congenital deficiency of TC II (7). Many types of cells are depleted to similar degrees in PA, but the Cbl deficiency of TC II deficiency, while profound, appears to be limited to rapidly dividing cells such as those of the hematopoietic system. Therefore, the TC II deficiency might have a greater, but more selective, impact on the function of the lymphocyte than occurs in PA.

Much is known about the Cbl metabolism of the lymphocyte, but the relationships with Ig production have not been studied. TC II promotes the entry of Cbl into human lymphocytes (8). Uptake and conversion of the Cbl to active forms are enhanced by stimulation by mitogens (9,10). Transformed lymphocytes from subjects with Cbl deficiency show megaloblastic changes similar to those of other blood cells (11). Deficient lymphocytes have been reported to take up Cbl less well (9) and better than (10) normal cells and to convert Cbl added in vitro more readily (10). Transformation by mitogens has been reported to be the same as in normal cells (11) and depressed (12), although techniques differed. In the latter study, transformation was measured by incorporation of ^{3}H thymidine and it was concluded that blastogenesis of PA lymphocytes was not impaired, but there was a failure of intranuclear incorporation of the label. The cells for these studies (12) were taken from patients with PA in remission and should not have been Cbl deficient. In another study (11) the biochemical lesion of Cbl deficient lymphocytes resembled that of other dividing Cbl deficient cells. Incorporation of [^{3}H] thymidine into DNA was greater than normal, with a sub-normal inhibition of deoxyuridine, partial correction by Cbl in vitro and complete correction by folic or folinic acid.

Thus, the studies of Cbl deficient lymphocytes have been directed, and with conflicting observations, toward abnormalities of transformation and division rather than to Ig synthesis. In the present study we measured IgG synthesis by human lymphocytes either collected from Cbl deficient persons or depleted in vitro.

MATERIALS AND METHODS

Subjects. The normal human lymphocytes (PBL) were collected from healthy paid donors. The 5 subjects with Cbl deficiency were all severely depleted, the serum Cbl by bioassay with E. gracilis being 16-69 pg/ml. Longstanding partial gastrectomy was the etiology in two and pernicious anemia in the others. There was a mixture of clinical expressions from predominately hematologic to mainly neurologic. Informed consent was obtained and the study conducted under the approval of the institutional committee overseeing human studies.

Collection of Lymphocytes. Forty ml of venous blood was collected in vacutainers containing heparin. The basic separation technique was that of Boyum (13) with minor modifications. The separated cells were washed twice with 0.99% sterile NaCl, counted manually, and suspended in RPMI 1640 culture media with either 5% fetal calf serum (FCS) or 0.7 g/dl of bovine serum albumin (BSA) depending on the nature of the experiment. All media contained 100 units of penicillin and 100 µg of streptomycin per ml.

Manipulation of Cbl and Folate Status of the Cells. There were two lines of approach, (a) the comparison of cell transformation, replication, and synthesis of IgG and total protein between cells derived from normal donors and those from Cbl deficient persons. Also studied were the effects of Cbl added in vitro on all measurements in both types of cells. The media for these studies contained 5% FCS and 1.0 µg/ml of pteroylglutamic acid (PGA) CN Cbl

was either withheld or added in amounts of 5.0 ng/ml. (b) The above measurements in normal PBL after depletion by culturing for 6 d in RPMI 1640 media containing BSA and no FCS or Cbl.

Conditions of Culture. With the exception of some preliminary studies described below, all cells were stimulated with reconstituted pokeweed mitogen (PWM) 0.1 ml/10 ml media, added at the start of culture. All cells were cultured at a concentration of $5x10^5$/ml in a total volume of 10 ml in 125 ml Ehrlenmeyer flasks with cotton plugs. They were maintained in a CO_2 incubator, 5% CO_2, 95% air for six days.

At harvesting, the cells were counted manually with Trypan Blue in the diluent in order to identify dead cells. An aliquot was centrifuged in a cytostat, the cells were stained and the percent transformed determined visually.

Measurement of Protein and IgG Synthesis. These measurements were made only on the media from the cultures and not the cells. Incorporation of ^3H leucine into total protein and IgG during 4 hrs at 37°C was measured by the method of Choi (14). The total protein fraction was precipitated by 10% trichloroacetic acid. 50 µg of IgG were added to another aliquot and an amount of the above anti human IgG to precipitate twice this amount was then added. The human IgG used as a reagent showed single bands on immunoelectrophoresis with anti human serum and anti human IgG, but none with anti human IgA or IgM. Antibody against this IgG was produced in rabbits and the immunoglobulin fraction isolated by precipitation at 50% saturation $(NH_4)_2SO_4$ followed by chromatography with DEAE cellulose.

Preliminary Studies. Although it was assumed from reports in the literature that Cbl enters PBL and that entry is enhanced by mitogens, some pertinent studies were repeated prior to the main experiments. The Cbl uptake studies were conducted as described for HeLa cells and for established lines of lymphocytes (15). The TC II for these studies was partially purified from Cohn Fraction III (15). The forms of Cbl within the lymphocytes were measured by chromatography with SP-Sephadex C-25 as before (16).

MATERIALS

RPMI 1640 media (without L-glutamine, folic acid or CN Cbl); minimum essential media (MEM-Eagle's, without leucine); penicillin; streptomycin; pterolyglutamic acid (folic acid, PGA) and L-glutamine from Grand Island Biological Co., Grand Island, N.Y. Both mitogens from the same company, Pokeweed Mitogen (PWM), Catalogue #670-5360, and phytohemagglutinim, #670-0576, were each reconstituted to 5 ml for use. Fetal calf serum (FCS) from Flow Laboratories, Rockville, Maryland. Cyanocobalamin (CN Cbl); Ficoll (Type 400); bovine serum albumin (Fraction V BSA) and Sigmacote (silicone) from Sigma Chemical Co., St. Louis, Missouri. Hypague sodium, 50% from Sterling Wintrop Products, Rensselaer, N.Y. CN [^{57}Co] Cbl with a specific activity of about 200 µCi/µg from Amersham Corp., Arlington Heights, Ill. was diluted with CN Cbl to give a specific activity of 20-40 µCi/µg for uptake studies. Tritiated L-Leucine (L-[4,5-^3H (N)]), specific activity 40-60 Ci/mmol, from New England Nuclear, Boston, Mass. Human IgG from Miles Laboratories, Elkhart, Indiana.

RESULTS

Preliminary Studies. The uptake of CN [^{57}Co] Cbl was measured in a series of experiments with normal peripheral blood lymphocytes which had been cultured for three days in complete media with FCS, CN Cbl and PGA, but either with or

without a mitogen. Transcobalamin II (TC II) enhanced uptake by stimulated cells in a pattern resembling that of other cell types studied previously, Table I.

TABLE I
Uptake of Free vs TC II Bound CN [^{57}Co] Cbl by PBL*

Cbl in Culture ng/10^6 cells	Cbl Uptake - pg/10^6 Cells	
	Free Cbl	TC II-Cbl
0.5	0.6	6.0
1.25	1.1	7.1
2.0	1.7	8.6
2.75	2.0	8.0
10.0	11.6	13.1

*Cells had first been cultured in complete media for 3 d with PWM. Uptake, measured in duplicate, proceeded for a period of 3 hrs at 37°.

In several experiments with varying concentrations of Cbl and time, there was the start of a plateau effect at a CN Cbl concentration of 1.0 ng/10^6 cells and at 3 hrs incubation at 37°. Stimulation with mitogens enhanced uptake of TC II-Cbl. For example, in a typical set of studies the cells were exposed to 2 ng of TC II-CN [^{57}Co] Cbl for 3 hrs at 37°. The uptake, in pg/10^6 cells, in cells cultured 3 d without mitogens was 1.65. Where PWM was present, the uptake was 3.14 pg Cbl/10^6 cells and with PHA, 6.53. Doubling the amount of PHA from 0.1 ml/10 ml to 0.2 ml had no added effect.

For one experiment, 30x10^6 cells were exposed to 60 ng of TC II-CN [^{57}Co] Cbl for 3 hours at 37°, harvested, lysed and the supernatant subjected to gel filtration on Sephadex G-200. Within the cell were 27 pg of free [^{57}Co] Cbl, 86 pg bound to a substance the size of TC II and 36 pg to the large molecular weight binder. In other cell types this pattern has corresponded to (a) penetration of TC II-Cbl into the cell, (b) release of some free Cbl, (c) beginning conversion of CN Cbl to coenzyme Cbl, and (d) complexing with apo enzymes.

In a second, similar experiment the CN Cbl exposure of the main studies was reproduced. 5x10^6 PBL from a normal donor were cultured for 6 d in media containing FCS, PGA and 5 ng of CN [^{57}Co] Cbl per 5x10^5 cells. The cells were lysed, the Cbl extracted and separated as before (16). A total of 7.4 pg [^{57}Co] Cbl was recovered in the 4 Cbl peaks. During culture, the free Cbl penetrated the cell and was converted to both Me Cbl and Ado Cbl, Figure 1. Proportionally there was more Me Cbl than observed in similar conversion by RMPI 6410 lymphoblasts (16). However, the present cultures were much longer in duration and the Me Cbl may require more time to accumulate. There was no definite peak of OH [^{57}Co] Cbl but the SO_3 [^{57}Co] Cbl observed may have been derived from the OH form.

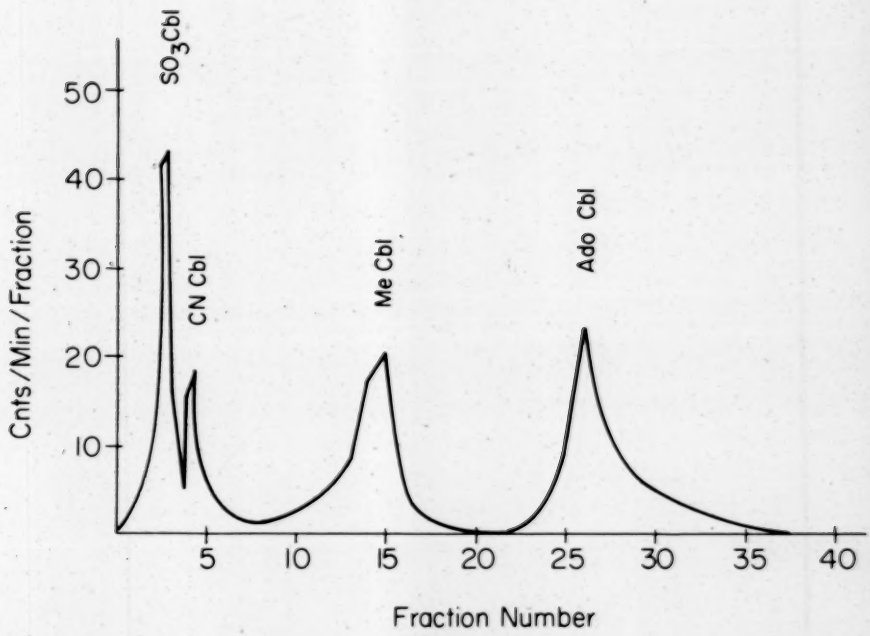

FIG. 1

Separation of the labeled cobalamins in lysed PBL that had been cultured for 6 days in media containing 5 ng of free CN [^{57}Co] Cbl/5x10^5 cells. The SP-Sephadex C-25 column was marked with a mixture of the individual Cbls (not illustrated). The 4 peaks contained respectively SO_3-Cbl - 3.7 pg; CN-Cbl - 1.6 pg; Me Cbl - 1.2 pg; and 0.9 pg of Ado Cbl.

The above studies were conducted to show that lymphocytes do take up Cbl and that the process is enhanced by TC II as in other types of cells. We deviated from some of these conditions in the performance of the main experiments of this study. PHA induced a greater Cbl uptake than PWM, but at the time our studies were started, PWM was considered to be the mitogen of choice in the induction of Ig production. In fact, in a single experiment, protein and IgG synthesis were twice as great after PWM stimulation than with PHA (data not shown). Although physiologically Cbl probably enters the PBL bound to TC II, we excluded any human TC II from the cultures of the experiments described below. We wanted to avoid the effects of constituents of human serum other than Cbl and therefore added Cbl in free form. Some free Cbl, when supplied in large amounts will be taken up by the cells, Table I, and converted to active coenzymes as shown above for PBL and earlier for other types of cells (17,18).

Stimulated Lymphocytes from Normal and Cbl Depleted Subjects. Initially, measurements were made on d 3 as well as d 6. Cell numbers did not increase until after d 3 and protein synthesis had not reached a maximum by d 3. In some preliminary studies the synthetic capacities of the cells were well developed by d 6, although the maximal accumulation of IgG as detected by RIA was not achieved until later. Considering all factors, d 6 was selected for termination of the cultures. There was a 28-40% net increase in cell numbers during the 6 days of culture in the presence of FCS, PGA, and PHA. From one third to three fourths of the final cells were identified as transformed by morphologic

examination. Neither cell number nor percent transformed differed between cells from control and Cbl deficient subjects and the added Cbl had no significant effect in either group, Table II.

TABLE II

6 Day Cell Counts and ^{3}H Leucine Incorporations
For Lymphocytes from Healthy and Cbl Depleted Subjects

	Cell Counts Day 6*			
	Mean ± SD (cells x 10^5/ml)*		% Blasts	
	No Cbl	Plus Cbl	No Cbl	Plus Cbl
Controls (5)	6.4 ± 1.8	6.5 ± 2.8	49	42
Cbl Deficient (5)	6.8 ± 2.1	7.0 ± 1.7	59	58

	Protein Synthesis Day 6 Mean c/m ± SD			
	Total Protein		IgG	
	No Cbl	Plus Cbl	No Cbl	Plus Cbl
Controls (5)	32.5 ± 8.4	34.1 ± 8.9	13.1 ± 14.5	15.3 ± 12.7
Cbl Deficient (5)	24.5 ± 14.7	29.9 ± 15.2	5.3 ± 3.7	7.5 ± 5.5

*Started with $5x10^5$/ml

Although total protein and IgG synthesis and release as measured by ^{3}H leucine incorporation by control cells were greater, the differences did not reach significance because of wide variations among cells from different donors, Table II and Figures 2 and 3.

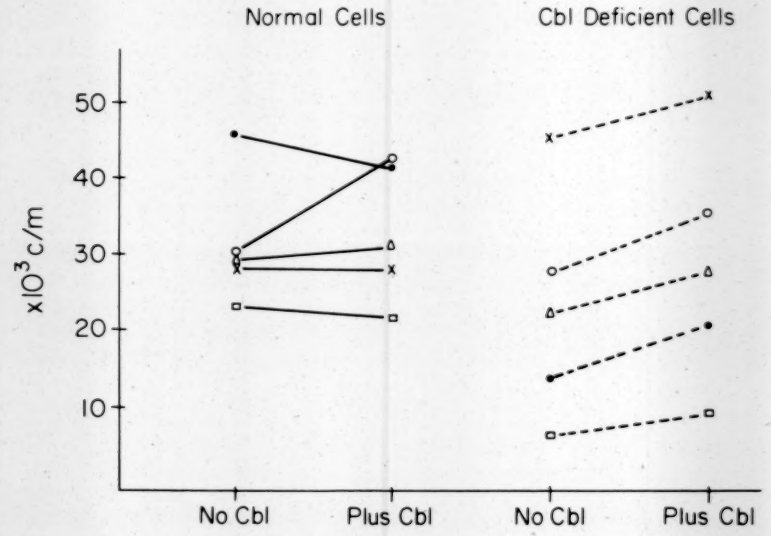

FIG. 2

Total protein synthesis and release by cells from healthy and depleted subjects on d 6 of culture as measured by ^{3}H leucine incorporation. Illustrated are activities of cultures of cells from same subjects with or without 5.0 ng/ml of added CN Cbl.

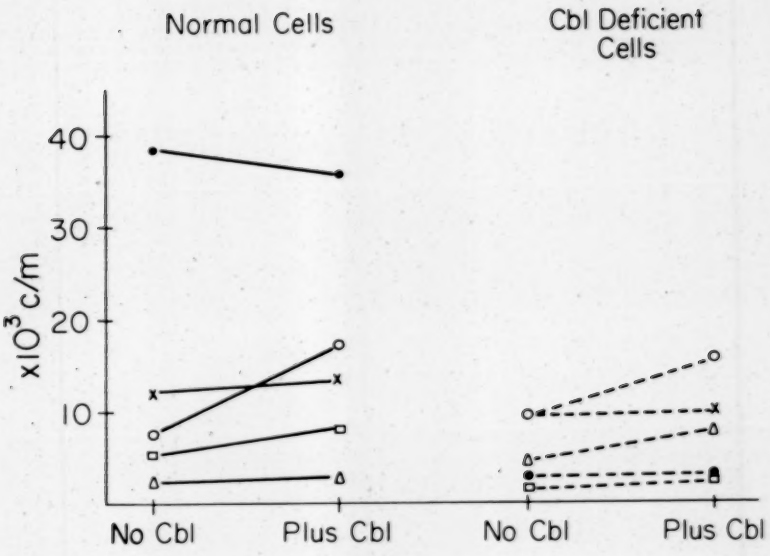

FIG. 3

IgG synthesis and release under the same circumstances of the experiments of Figure 2.

Cbl added in vitro had no significant effect on protein or IgG synthesis by control cells from normal donors. However, as evaluated by the t test for matched pairs, the enhancement of total protein synthesis of cells from Cbl deficient persons by added Cbl was significant, $p < 0.001$. Although added Cbl also enhanced IgG synthesis in each case, Figure 3, the change did not quite reach significance.

Peripheral Human Cells Depleted In Vitro. In an attempt to avoid reliance upon the scarce Cbl deficient person as a source of depleted cells, we established an in vitro model of depletion. The strategy was to avoid any source of Cbl other than that in the PBL as collected. The fetal calf serum (FCS) was a potential source of Cbl and TC II, although Sauer (19) could deplete an established line of human lymphoblasts cultured with FCS. The FCS used in the present studies contained 226 pg Cbl/ml as assayed by Euglena gracilis. At 5% FCS, a routine 10 ml culture thus contained 113 pg Cbl. Another lot was examined more extensively, Table III. All of the endogenous Cbl in this lot, 115-128 pg/ml, was bound to TC II, and there was an equal amount of apo TC II. The most abundant apo carrier was a substance of the apparent molecular size of human R binder of Cbl, but it carried no endogenous Cbl. There were lesser amounts of an apo binder larger in size. The small amount of TC II-Cbl in FCS, and added Cbl bound to apo TC II, could be available to the cells, if calf TC II will function in a human system. Whereas we have tested the function of the TC II of several species in the model based on RPMI 6410 lymphocytes of human origin (20), we did not include bovine TC II.

TABLE III
Cbl Content and Binding in Fetal Calf Serum
all data as pg/ml

Cbl Content, E. gracilis Assay			Cbl Binding Capacity by Gel Filtration			
Total	TC II-Cbl*	R-Cbl*	Total	Vo	TC II	R
115	128	0	1408	251	159+	998

*By bioassay of fully saturated components as separated by gel filtration; contribution of added ^{57}Co Cbl then subtracted.

+Level by precipitation with 1.96 M $(NH_4)_2SO_4$ was 155 pg/ml.

The next step was to study the replacement of FCS with 7 mg/ml of bovine serum albumin (BSA), a technique used by Fujii et al to deplete mouse leukemic cells of Cbl in vitro (21). First, we measured the amount of Cbl by bioassay in PBL under varied conditions of culture, including replacement of the FCS with BSA, Table IV.

TABLE IV

Cbl Content as pg/10^6 Cells
After 6 Days of Culture of Normal Human Lymphocytes*
Under Varied Conditions

"Serum" added	Cbl added	Pg Cbl/10^6
Pre Culture		1.5
FCS	5 ng/ml	8.9
FCS	0	3.0
BSA	5 ng/ml	5.3
BSA	0	2.2

*Total cells per culture 10-20 x 10^6. The data are derived from the four individual cultures of a single collection of cells.

The BSA used in these depletion studies was free of Cbl. The Cbl content of the cells was so low that the limits of the assay were approached, but some interpretation may be possible. Apparently upon transformation with a mitogen, cell division and induction of function, the PBL will take up Cbl. The amount of Cbl in the surroundings seems to be a limiting factor. Thus, culturing PBL in a medium free of Cbl in any form, including that in any serum product, creates an at least partial deficiency.

Through several preparatory studies it was then determined that PBL from healthy donors would multiply, transform and synthesize protein when cultured for 6 days in the presence of BSA, but no FCS. (Data not shown). This model was applied by culturing six lots of cells from normal donors in duplicate or quadruplicate for 6 days in media containing BSA, PGA but no FCS. There was blastogenesis, cell replication and protein synthesis, Table V. Half of the cultures from a given donor contained 5 ng of Cbl per ml while there was none in the others. The Cbl status was the only difference between pairs. Although cell growth was slightly greater in the presence of Cbl, the difference was not significant. Cbl significantly increased the ^3H leucine

incorporation into total protein, Table V and Figure 4. (t test for matched pairs gave a $p < 0.05$). Cbl also increased IgG synthesis, Figure 4 and Table V, but there was a wide range of values and the difference did not reach significance.

TABLE V

6 Day Cell Counts and ^3H Leucine Incorporations for Lymphocytes Collected from 6 Healthy Subjects, but Cultured in Cbl-Free Media

	Cell Counts - Day 6	
	Mean ± SD Total Cells x 10^5/ml*	Blasts %
Plain Media	5.76 ± 1.23	35
Media plus 5 ng CN Cbl/ml	5.85 ± 1.96	37

	Protein Synthesis - Day 6 Mean c/m ± SD	
	Total Protein	IgG
Plain Media	36.9 ± 23.2	12.4 ± 12.2
Media plus 5 ng CN Cbl/ml	53.7 ± 26.0	23.3 ± 17.5

*Started with 5x10^5ml

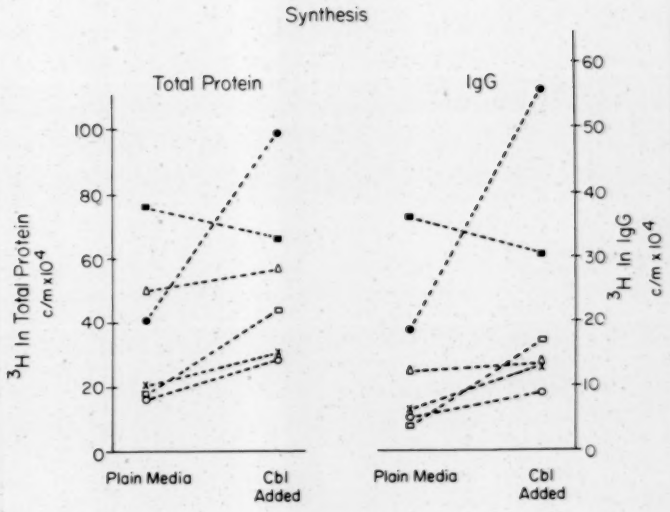

FIG. 4

Total protein and IgG synthesis and release by cells from healthy donors but cultured for 6 days, with mitogens, in media containing BSA, PGA, but either no Cbl or 5 ng Cbl/ml. The paired samples are aliquots from the same donor and the same symbol is used for a given donor in both sections of the plot.

DISCUSSION

The present study gave further support to the thesis, outlined in the Introduction, that Cbl enters the PBL but principally at some point during the process encompassed by transformation. TC II enhanced uptake of cells stimulated by mitogens, confirming an earlier (8) but little studied observation. Free Cbl, with exposure to larger amounts, also entered cells and was converted to coenzymes.

Cbl enhanced protein synthesis and release by cells from patients deficient in Cbl. IgG synthesis was invariably increased by Cbl in the same cells, but the change did not reach statistical significance; there was a wide range in rates of synthesis among cells from different donors. Both protein and Ig synthesis were increased by Cbl in cells restricted in Cbl uptake during culture.

The mechanisms behind the present observations remain unknown. Since DNA synthesis by at least some types of bone marrow cells is impaired in Cbl deficiency (22) the total Ig output of a Cbl deficient person could be suppressed by a failure of formation of an adequate lymphocyte mass in response to a stimulus. The present observations do not support that concept, although it should be explored further. In vitro, both cells from Cbl deficient persons and those rendered deficient during culture reproduced and were transformed by mitogens to the same degree as replete cells. However, the techniques used here were incomplete. We measured only net cells at the end of a 6 d culture and not the number of cell divisions. Transformation was measured by the somewhat crude visual approach. In order to keep procedures simple and to divert all available cells to the measurement of synthesis, we did not apply more sophisticated methods. Had the simple approach suggested abnormalities of growth and transformation, we would have explored further, but they did not.

The effect of Cbl, free or TC II bound, on lymphocytes may be complex. Protein synthesis is not uniform throughout the cell cycle (23) and Cbl could influence the phase of the cells. Such an effect might explain the apparent suppression of protein and IgG synthesis in one set of cells of the study of Figure 4. All of the experiments here were performed in duplicate or quadruplicate. Invariably all cultures from a given donor on a given day responded to Cbl in an identical fashion. This was true of the 4 cultures from one collection that reacted to Cbl by a decreased synthesis. Ig synthesis was measured here by leucine incorporation by cells and then release which measures the capacity of the cells at the moment of exposure to the leucine. Cumulative IgG production can be measured by a radioimmunoassay (RIA) of the media, but published RIAs were not sensitive enough for the present study. We have devised an RIA which will just measure the IgG released and with further technical modification will be applicable to the type of study presented here.

Cbl appeared to have some direct function in protein synthesis, not obviously reflected in transformation and replication. Yoshida et al did not find depressed protein synthesis in cells of Cbl deficient bone marrow (24). They did not study in vitro activity of Cbl, the most striking observation of the present study. The relationships between Cbl and protein synthesis, particularly Ig synthesis by lymphocytes, has been largely ignored. Cbl dependent homocysteine-methionine methyltransferase is found in PBL (25) and in established human lymphoblastoid lines (19). (In unpublished studies we have duplicated both observations.) Conceivably the reaction catalyzed by this enzyme, the products of which may participate in methylations, may be involved in the IgG synthesis studied here. There is an interesting line

of parallel research which should be mentioned. In adenosine deaminase deficiency, which is characterized by severe combined immunodeficiency, there may be accumulation of S-adenosylhomocysteine and inhibition of methylation via S-adenosylmethionine, (26,27). The latter participates directly in several methylations and in the Cbl dependent homocysteine-methionine transmethylation. Some of the potentially affected methylations may be required for protein synthesis by the lymphocyte (27).

In the present study the parallel declines and increases in protein and IgG synthesis suggested that the involvement of Cbl was at a stage of the synthesis of IgG common to both. The depression of IgG synthesis did seem to be somewhat more profound than the decrease in total protein synthesis in cells from subjects depleted of Cbl in vivo. However, the fraction of protein synthesis contributed by IgG was not depressed in cells depleted in vitro and in none of the studies did the addition of Cbl change this fraction, which averaged 36% in normal cells. Although the capacity of cells from deficient donors to synthesize IgG was half that of cells from normal donors, (Table II), the impairment did not reach statistical significance. The trend suggested that with accumulation of more cases, a significant impairment would characterize the group or at least cells from certain individual subjects.

ACKNOWLEDGEMENTS

The authors wish to express their appreciation to Dr. Anthony P. Tartaglia and Dr. Peter T. Burkhart of St. Peters Hospital, Albany, NY and to the staff of the Neurology Service of the Albany Veterans Administration Medical Center for the referral of Cbl deficient patients. We especially thank Mr. Thomas A. Morelli, Mr. Joseph A. Manzi and Ms. Lisa S. Lazzaro for their contributions to different phases of this work. This work was supported by USPHS Grant 5 R01 AM17767-07 and by the Veterans Administration.

REFERENCES

1. HITZIG, W.H. and KENNY, A.B. The Role of Vitamin B_{12} and Its Transport Globulins in the Production of Antibodies. Clin. Exp. Immunol. 20: 105-111, 1975.

2. FRATER-SCHRODER, M., SACHER, M., and HITZIG, W.H. Inheritance of Transcobalamin II (TC II) in Two Families with TC II Deficiency and Related Immunodeficiency. J. Inherit. Metab. Dis. (In press).

3. NEWBERNE, P.M. Effect of Folic Acid, B_{12}, Choline, and Methionine on Immunocompetence and Cell-Mediated Immunity. In: Malnutrition and the Immune Response. R.M. Suskind (ed). Raven Press, New York, 1977, pp. 375-386.

4. vanDOMMELEN, C.K.V., SLAGBOOM, G., MEESTER, G.T., and WADMAN, S.K. Reversible Hypogammaglobulinaemia in Cyanocobalamin (B_{12}) Deficiency. Acta Med. Scand. 174: 193-200, 1963.

5. CONN, H.O., BINDER, H., and BURNS, B. Pernicious Anemia and Immunologic Deficiency. Ann. Int. Med. 68: 603-612, 1968.

6. HIPPE, E., and JENSEN, K.B. Hereditary Factors in Pernicious Anaemia and Their Relation to Serum-Immunoglobulin Levels and Age at Diagnosis. Lancet 2: 721-722, 1969.

7. HALL, C.A., The Plasma Transport of Cobalamin (Cbl). In: Vitamin B_{12}. B. Zagalak, and W. Friedrich (eds). Walter deGruyter & Co., Berlin, 1979, pp. 725-742.

8. MEYER, L.M., MILLER, I.F., GIZIS, E., TRIPPE, E., and HOFFBRAND, A.V. Delivery of Vitamin B_{12} to Human Lymphocytes by Transcobalamins I, II, and III. Proc. Soc. Exp. Biol. Med. 146: 747-750, 1974.

9. HOFFBRAND, A.V., TRIPPE, E., and DAS, K.C. Uptake of Vitamin B_{12} by Phytohaemagglutinin-Transformed Lymphocytes. Br. J. Haematol. 24: 147-156, 1973.

10. QUADROS, E.V., MATTHEWS, D.M., HOFFBRAND, A.V., and LINNELL, J.C. Synthesis of Cobalamin Coenzymes by Human Lymphocytes In Vitro and the Effect of Folates and Metabolic Inhibitors. Blood 48: 609-619, 1976.

11. DAS, K.C., and HOFFBRAND, A.V. Lymphocyte Transformation in Megaloblastic Anaemia: Morphology and DNA Synthesis. Br. J. Haematol. 19: 459-468, 1970.

12. MacCUISH, A.C., URBANIAK, S.J., GOLDSTONE, A.H., AND IRVINE, W.J. PHA Responsiveness and Subpopulations of Circulating Lymphocytes in Pernicious Anemia. Blood 44: 849-855, 1974.

13. BOYUM, A. Isolation of Leukocytes from Human Blood. A Two-Phase System for Removal of Red Cells with Methyl Cellulose as Erythrocyte Aggregating Agent. Scand. J. Clin. Invest. 21: 9-29, 1968.

14. CHOI, Y.S. Serological Precipitation Methods for Studying Biosynthesis and Secretion of Immunoglobulins by Human Peripheral Lymphocytes. J. Immunol. Methods. 14: 37-40, 1977.

15. HALL, CHARLES A., and GREEN, PAMELA D. Competition Between Apo and Holo Transcobalamin II (TC II) for the TC II-Mediated Uptake Process. Proc. Soc. Exp. Biol. Med. 158: 206-209, 1978.

16. BEGLEY, J.A., and HALL, C.A. Overestimation of Cyanocobalamin Due to Coelution of Sulfitocobalamin on SP-Sephadex C-25. J. Chromatography 177: 360-362, 1979.

17. HALL, C.A., HITZIG, W.H., GREEN, P.D., and BEGLEY, J.A. Transport of Therapeutic Cyanocobalamin in the Congenital Deficiency of Transcobalamin II (TC II). Blood 53: 251-263, 1979.

18. BERLINER, NANCY and ROSENBERG, LEON E. Uptake and Metabolism of Free Cyanocobalamin by Cultured Human Fibroblasts from Controls and a Patient with Transcobalamin II Deficiency. Metabolism 30: 230-236, 1981.

19. SAUER, H. Cobalamin-Dependent Methionine Synthesis and the Regulation of the Metabolism of Activated Folic Acid Derivatives in Cultured Human Lymphoblasts. In: Vitamin B_{12}. B. Zagalak and W. Friedrich (eds). Walter deGruyter & Co., Berlin, 1979, pp. 1145-1148.

20. HAUS, M., GREEN, P.D., and HALL, C.A. Species Specificity in the Immunologic Reactions and Biological Functions of Transcobalamin II. Proc. Soc. Exp. Biol. Med. 162: 295-298, 1979.

21. FUJII, K., NAGASAKI, T., and HUENNEKENS, F.M. Vitamin B_{12} Requirement for Replication of Mouse Leukemia L1210 Cells: Functional Relevance to Folate Metabolism. In: Vitamin B_{12}. B. Zagalak and W. Friedrich, (eds). Walter deGruyter & Co., Berlin, 1979, pp. 1127-1130.

22. BECK, WILLIAM. Metabolic Features of Cobalamin Deficiency in Man. In: Cobalamin: Biochemistry and Pathophysiology. Bernard M. Babior (ed). John Wiley & Sons, New York, 1975, pp. 403-450.

23. BUELL, D.N., and FAHEY, J.L. Limited Periods of Gene Expression in Immunoglobulin-Synthesizing Cells. Science 164: 1524-1525, 1969.

24. YOSHIDA, Y., TODO A., SHIRAKAWA, S., WAKISAKA, G., and UCHINO, H. Proliferation of Megaloblasts in Pernicious Anemia as Observed from Nucleic Acid Metabolism. Blood 31: 292-303, 1968.

25. PEYTREMANN, R., THORNDIKE, J., and BECK, W.A. Studies on N^5-Methyl-tetrahydrofolate homocysteine Methyltransferase in Normal and Leukemic Leukocytes. J. Clin. Invest. 56: 1293-1301, 1975.

26. KREDICH, N.M., and MARTIN, D.W. Jr., Role of S-Adenosyl-homocysteine in Adenosine-Mediated Toxicity in Cultured Mouse T Lymphoma Cells. Cell 12: 931-938, 1977.

27. HERSHFIELD, M.S., and KREDICH, N.M. In Vivo Inactivation of Erythrocyte S-Adenosylhomocysteine Hydrolase by 2'-Deoxyadenosine in Adenosine Deaminase-Deficient Patients. J. Clin. Invest. 63: 807-811, 1979.

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NUTRITION RESEARCH, Vol. 1, pg. 363-372, 1981
0271-5317/81/040363-10$02.00/0 Printed in the USA.
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COPPER AND IRON DEFICIENCIES: EFFECTS ON SERUM LIPIDS AND TISSUE MINERALS

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ABSTRACT

To study the interrelationship between iron and copper on serum lipid concentrations, four diets were fed to growing rats: iron and copper deficient, copper-deficient, iron-deficient, iron and copper adequate. After 18 weeks, concentrations of iron and copper in organs and lipids in sera were determined. Iron deficiency alone or combined with copper deficiency resulted in reduced body weights, hemoglobin concentrations, hematocrits, and iron concentrations in liver, spleen, and heart. Hepatic copper was elevated 8-fold in iron deficiency. Copper deficiency alone or combined with iron deficiency resulted in reduced copper concentrations of liver and spleen and reduced ceruloplasmin. Serum triglycerides and cholesterol did not differ among experimental treatments. No significant effects of the interaction between dietary iron and copper on serum lipid levels were found.

KEY WORDS: Iron, Copper, Iron-copper Interaction, Lipids

INTRODUCTION

The purpose of this study was to investigate the interrelationship between dietary iron and copper and its effect on serum lipid levels in rats. Previous work by the author and others has documented a hyperlipidemia of iron deficiency (1-5). In one of these studies, an accumulation of copper in livers of iron-deficient rats was observed (6). There is also a well-known accumulation of iron during copper deficiency in rats and pigs reported in the literature (7). Furthermore, increased serum cholesterol concentration has been associated with dietary copper deficiency (8-13). The question which then arises is the subject of this paper: Is the interrelationship between iron and copper responsible for changes in serum lipid concentrations?

Aberrations in lipid metabolism during both iron and copper deficiencies have been reported. Iron deficiency in rats and chicks has been related to elevated serum lipid levels and increased lipid synthesis (1-5). The hyperlipidemia in offspring of iron-deficient rats (1, 5) has been characterized by increased triglycerides, cholesterol, and phospholipids in serum and increased triglycerides in liver and is associated

with an increased incorporation of (U-^{14}C) glucose into triglycerides and CO_2 *in vitro* (5) and a decreased zinc:copper ratio in the liver (6). Normolipidemic iron-deficient adult rats have been reported to have increased triglyceride synthesis from $^{3}H_2O$ and glucose in adipose tissues, and increased synthesis of polar lipids from glucose in liver slices (14). An accumulation of copper in tissues of iron-deficient rats in utero, during suckling, and after weaning (15) parallels the increase in serum lipid concentrations (16).

Allen and Klevay (8) observed hypercholesterolemia and increased *in vitro* synthesis of cholesterol from (^{3}H)-mevalonate in rats fed copper-deficient diets for 181 days. Lei (9, 10, 12) suggests that during copper deficiency there is a shift of cholesterol from the liver to the serum pool or reduced rate of movement of cholestrol from serum which is responsible for the hypercholesterolemia. Petering *et al.* (11) have reported an inverse relationship between serum concentrations of copper or ceruloplasmin and cholesterol, triglycerides, and phospholipids in rats fed different levels of copper and zinc in drinking water.

In addition to iron and copper affecting lipids, iron and copper are known to interact with each other. As early as 1928, it was reported that copper-deficient rats became anemic due to impaired iron mobilization (7), suggestive of a role of copper in hemoglobin formation. Since then, it has been suggested that the mobilization of iron from liver for hemoglobin synthesis involves the conversion of ferrous to ferric iron, catalyzed by the copper-containing protein, ceruloplasmin (7, 18-21). Supporting this, Evans *et al.* (18) reported that growing rats deprived in copper accumulated hepatic iron in conjunction with a decrease in ceruloplasmin activity. Upon repletion with dietary copper, ceruloplasmin activity increased, liver iron decreased, and increases in hemoglobin and hematocrit levels followed.

The effect of iron on copper metabolism is less clear and few studies have considered this interaction. Sourkes *et al.* (22) have found a three-fold accumulation of copper in the livers of young iron-deprived rats only after seven to eight weeks of deprivation and abnormally high hepatic iron (2.8-fold increase) in those maintained on a copper-deficient diet, beginning four weeks after initiation of the dietary regimen. Owen (23) found that rats fed an iron-deficient diet for less than six months had an elevated uptake of copper and a reduced release of ceruloplasmin from the liver, whereas a more prolonged deficit of iron had little effect on copper. Similarly, Gregoriadis *et al.* (24) reported that rats fed an iron-deficient diet for 40, 50, and 90 days had increased hepatic copper after six to eight weeks.

None of these investigations has studied possible interrelationships between iron and copper and its effects on lipid metabolism. The research reported here studied these interrelationships.

MATERIALS AND METHODS

Female, Sprague-Dawley CD rats (Harlan Industries, Indianapolis, IN) were housed in individual stainless steel suspended cages in a temperature-controlled room (20°) in which a 12-hour light-dark cycle was maintained. The animals, weighing approximately 87 grams, were randomly assigned to one of 4 experimental groups and fed the appropriate diet and glass-distilled water ad libitum for 18 weeks. A 2 x 2 factorial design was employed--low

and adequate levels of iron and copper were fed. The experimental diets (Table 1) differed only in iron and copper contents and met or exceeded the National Research Council (NRC) requirements for all nutrients except iron and copper (25). The mineral mix was formulated to provide minerals at, or slightly above, the level suggested by the NRC.

TABLE 1

Composition of Experimental Diets

	Percent
Casein[1]	20.0
Sucrose	30.0
Cornstarch	31.3
Corn Oil	10.0
Vitamin Mix[2]	1.0
Mineral Mix[3]	3.7
Cellulose[4]	2.0

1. Vitamin-free test casein, Teklad, Madison, WI.

2. Teklad. Supplies in mg per kg of diet when added at 1% of diet: p-aminobenzoic acid, 110.1; ascorbic acid, 991.2; biotin, 0.4; vitamin B_{12}, 0.03; calcium panthothenate, 66.1; choline 1433.7; folic acid, 2.0; inositol, 110.1; menadione, 49.6; niacin, 99.1; pyridoxine-HCl, 22.0; riboflavin, 22.0; thiamine-HCl, 22.0. Supplies, in units per kg of diet, when added at 1% of diet: vitamin A-palmitate, 19,824; vitamin D_2, 2203; vitamin E acetate, 121. Cornstarch dilutent QS.

3. The iron and copper-free mineral mixture contained (per kg diet): NaCl, 3 g; KH_2PO_4, 13 g; Ca $(H_2PO_4)_2 \cdot H_2O$, 9 g; $CaCO_3$, 9 g; $MgSO_4$, 2.78 g; $MnSO_4 \cdot H_2O$, 169 mg; $CrK(SO_4)_2 \cdot 12H_2O$, 19.2 mg; KIO_3, 0.46 mg; $Na_2SeO_3 \cdot 5H_2O$, 0.34 mg; $ZnCO_3$, 28.8 mg; NaF, 2.2 mg. $FeSO_4 \cdot 7H_2O$ and $CuSO_4$ were added to the supplemented diets in place of cornstarch to provide 50 mg Fe/kg diet and 10 mg Cu/kg diet.

4. Cellulose supplied from, ICN.

The experimental diets were: (1) Iron and copper deficient, providing less than 1 ppm Cu and 5 ppm Fe; (2) Copper-deficient, providing less than 1 ppm Cu and 50 ppm Fe; (3) Iron-deficient, providing 5 ppm Fe, 10 ppm Cu; and (4) Control, providing 10 ppm Cu and 50 ppm Fe. Iron and copper contents of these diets were determined by atomic absorption spectrophotometry. To avoid mineral contamination, all materials used in contact with the rats or diets were stainless steel, glass, or plastic. All glassware was soaked in 50% HNO_3 and rinsed in glass-distilled water prior to use. Food consumption and body weights were determined weekly. At the end of the experimental period, tailblood samples were taken for hemoglobin and hematocrit determinations (26). After a 4-hour fast on the following day, the rats were briefly exposed to chloroform and as much blood as possible was collected by cardiac puncture. Livers, spleens, and hearts were excised using stainless steel instruments, rinsed in glass-distilled water, blotted dry and frozen for future analyses. Serum was recovered after centrifugation of blood and stored frozen.

Ceruloplasmin (EC1.16.3.1) activity was determined in serum by the method of Houchin (27) using human ceruloplasmin as a reference standard (Sigma Chemical Co., St. Louis, MO). Lipids were extracted from serum aliquots by the method of Folch et al. (28) and separated on Silica gel thin-layer chromatography plates (Merck) developed in a solvent system of petroleum ether: ethyl ether: acetic acid (70:30:1, V:V:V). Following iodine vapor exposure, lipid fractions were identified and recovered. Colorimetric assays were used to quantitate each of the lipid fractions. Triglyceride concentration was determined by the method of Stern and Shapiro (29), free and esterified cholesterol by the method of Searcy and Bergquist (30).

Organs were prepared for mineral analyses by drying at 100°C until a constant weight was obtained and then ashing at 550°C (Blue M Muffle Furnace) for approximately 12 hours. In the case of liver samples, a small amount of ash aide (20% KSO_4 in hot concentrated HNO_3) was added to charred samples to catalyze oxidation. The resulting ash was dissolved in several drops of HCl and brought to appropriate volume with glass-distilled water. Iron and copper contents were determined by atomic absorption spectrophotometry (Jarrell-Ash, Fisher Scientific Co.).

Two-way analysis of variance was used to determine statistically significant differences among group means (31).

RESULTS

Iron deficiency induced by feeding the Fe-deficient (group 3) diet or the Fe- and Cu-deficient diet (group 1) resulted in reduced body weights, hemoglobin concentrations, and hematocrits relative to both the Cu-deficient and control groups (Table 2). Although, copper deficiency produced lower CP levels in both groups fed the Cu-deficient diets (groups 1 and 2), it did not result in anemia or growth retardation.

Livers of iron-deficient rats in group 3 weighed less on an absolute basis than those of other groups (Table 3). However, when liver weight was expressed as a percent of body weight, there were no significant differences among groups. Reduced levels of iron were found in livers, spleens, and hearts of iron-deficient rats (groups 1 and 3). Hepatic copper concentrations were lowest in the two copper-deficient groups (1 and 2), and highest in the iron-deficient rats (group 3).

Iron deficiency (groups 1 and 3) was associated with a splenomegaly on an absolute basis and expressed as a percent of body weight. As expected, the severe deprivation of iron in groups 1 and 3 drained the splenic iron stores to less than 20% of normal. Dietary copper deficiency resulted in reduced splenic copper concentrations in rats of groups 1 and 2 relative to those of group 4. Total splenic copper levels were significantly lower in group 2 than in the other experimental groups. Spleens of iron-deficient rats (groups 1 and 3) had total copper contents which were not different from control rats (group 4). However, due to the splenomegaly of iron deficiency, the splenic copper concentrations in groups 1 and 3 were less than in group 4.

TABLE 2

Body Weights, Hemoglobin Levels, Hematocrits, and Serum Ceruloplasmin Activities of Rats After 18 Weeks on Experimental Diets

	Iron and Copper Deficient Group 1	Copper Deficient Group 2	Iron Deficient Group 3	Control Group 4
Body Weight (g)	236.6^{A}[1] ± 6.1	252.3^{B} ± 7.8	235.9^{A} ± 6.2	258.8^{B} ± 6.8
Hemoglobin (g/dl)	10.3^{A} ± 0.6	14.4^{B} ± 0.3	10.6^{A} ± 0.5	14.8^{B} ± 0.2
Hematocrit (%)	25.3^{A} ± 1.2	47.1^{B} ± 0.8	25.4^{A} ±2.1	47.1^{B} ±0.5
Ceruloplasmin[2] (units/ml)	4.27^{A} ± 0.32	5.13^{A} ± 0.20	35.1^{B} ± 2.51	37.06^{B} ± 2.11

[1]Mean ± SE, n = 7-9 rats/group. Means not sharing common superscripts are significantly different (Body weight, $p<0.01$; hemoglobin, hematocrit, ceruloplasmin, $p<0.001$). Statistical significance determined by two-way analysis of variance.

[2]Ceruloplasmin activity is expressed as units/ml serum. One unit is defined as the amount of "oxidase" activity which causes a A_{550} of 0.01 per minute at pH 5.5 at 37°C, using N_1N-Dimethyl-p-phenylenediamine as subtrate (27).

Iron deficiency alone or combined with copper deficiency produced cardiomegaly on an absolute basis and relative to body weight. Iron concentration was significantly decreased in hearts of rats in groups 1 and 3. This was due to a decrease in total heart iron and an increased size of the heart during iron deficiency. The rats deficient in both iron and copper had the lowest concentration and total iron content in the heart. The only significant difference in cardiac copper concentration among groups was that group 1 had less copper than group 3.

Serum lipid concentrations (Table 4) did not differ substantially among groups. No significant differences in triglyceride and free cholesterol concentrations were found among groups. Cholesterol ester and total cholesterol concentrations of sera from rats fed diets deficient in iron and copper were lower than those of the other dietary treatment groups.

TABLE 3

Iron and Copper Concentrations of Liver, Spleen, and Heart After 18 Weeks on Experimental Diets

	Iron and Copper Deficient Group 1	Copper Deficient Group 2	Iron Deficient Group 3	Control Group 4
Liver dry wt. (g)	2.33^{AB} [1] ± 0.09	2.47^{A} ± 0.18	1.94^{B} ±0.11	2.13^{AB} ±0.10
Iron (μg/g dry wt.)	169.7^{A} ±35.4	1006.9^{B} ± 206.2	161.3^{A} ± 18.2	967.0^{B} ± 51.0
Copper (μg/g dry wt.)	15.4^{A} ± 0.8	15.5^{A} ± 2.1	129.8^{B} ± 43.5	22.7^{C} ± 1.9
Spleen dry wt. (mg)	181^{A} ±17	123^{B} ± 5	180^{A} ± 10	124^{B} ± 6
Iron (μg/g dry wt.)	554.7^{A} ± 24.7	4471.6^{B} ± 687.8	620.3^{A} ± 29.1	3799.1^{B} ± 739.2
Copper (μg/g dry wt.)	24.9^{A} ± 2.2	28.1^{A} ± 1.9	28.3^{A} ± 1.6	44.5^{B} ± 6.4
Heart dry wt. (mg)	244^{A} ±15	206^{B} ±10	242^{A} ±10	205^{B} ±10
Iron (μg/g dry wt.)	275.6^{A} ± 8.7	421.8^{BD} ± 31.8	307.5^{C} ± 9.2	463.0^{D} ± 39.9
Copper (μg/g dry wt.)	24.9^{A} ± 2.6	$30.1^{A,B}$ ± 2.8	38.9^{B} ± 3.7	$32.3^{A,B}$ ± 2.9

[1] Mean ± SE, n = 7-9 rats/group. Means not sharing common superscripts are significantly different ($p < 0.05$) as determined by analysis of variance.

TABLE 4

Serum Lipid Concentrations of Rats After 18 Weeks on Experimental Diets

	Iron and Copper Deficient Group 1	Copper Deficient Group 2	Iron Deficient Group 3	Control Group 4
Triglycerides (mg/dl)	40.2[1] ± 5.6	28.5 ± 2.6	32.6 ± 4.9	33.0 ± 3.2
Cholesterol (mg/dl)	8.6 ± 0.7	13.5 ± 2.5	12.8 ± 2.3	11.1 ± 2.0
Cholesterol Ester (mg/dl)	28.1^{A} ± 4.7	52.6^{B} ± 9.8	42.9^{A,B} ± 6.9	43.2^{A,B} ± 8.7
Total Cholesterol[2] (mg/dl)	35.9^{A} ± 4.8	66.2^{B} ± 11.6	55.7^{A,B} ± 9.0	63.6^{B} ± 6.2

[1]Mean ± SE, n = 7-9 rats/group. Means not sharing common superscripts are significantly different ($p<0.05$). Statistical significance determined by analysis of variance.

[2]Total cholesterol is calculated from the sum of cholesterol + cholesterol ester.

DISCUSSION

These experimental results show that dietary deficiency of iron and combined iron and copper deficiencies are more severe and have more pronounced effects than copper deficiency alone. Retardation of growth and anemia were produced exclusively from iron deficiency. Group 1 (iron and copper deficient) had body weights, hemoglobin level and hematocrits comparable to group 3. Thus, it appears that iron was the limiting mineral for growth and hematologic status.

The most striking effects of the experimental treatments on tissue iron and copper concentrations were due to iron deficiency. Iron concentrations of liver and spleen were markedly reduced and that of heart moderately reduced in iron deficiency. An 8-fold increase in hepatic copper concentration was also found in iron-deficient rats. This confirms previous reports of Sourkes et al. (22), Owen et al. (23), Gregoriadis et al. (24), and Sherman et al. (15). However, the mechanism by which hepatic copper is increased in iron deficiency is not presently known. Differences in absorption, uptake, distribution, or tissue metabolism may be responsible for the abnormal accumulation of copper during iron deficiency. Previously, it was found that hepatic copper is elevated during gestational, suckling, and post-weaning iron deprivation (15). It was also found that in addition to an accumulation of copper during iron deficiency, the normal mobilization of copper from liver during post-natal development is impaired. We have speculated that the required levels of ceruloplasmin may be lowered during iron deficiency as a result of lowered ferroxidase needs and therefore excess copper is stored in the liver. The results presented here indicate that this is not the case since CP levels were not lower in iron deficiency. Further research is needed to identify the mechanism involved

in the accumulation of copper in livers of iron-deficient rats. In addition, there was a trend towards increased concentrations of iron in liver and spleen during copper deficiency as has been previously reported (22).

The normal concentrations of serum lipids in adult iron-deficient rats of this study confirm our previous work (14). The lack of significant elevations in serum cholesterol during the copper deficiency in this study does not confirm the work of Lei (9) and Allen and Klevay (8, 13). The lack of agreement between studies may be accounted for by differences in experimental design, including dietary levels of other minerals, age, sex, and severity of deficiency symptoms since varying degrees of copper depletion were present in all studies. For example, although the copper contents of the diets are comparable, Lei's studies utilized a diet providing 120 ppm zinc, whereas the diet in the present study contained 15 ppm zinc. Since high levels of dietary zinc depress organ copper levels and aggravate a copper deprivation (32), the high levels of dietary zinc in Lei's studies resulted in a more severe copper deficiency than that manifested in the present study. Copper-deficient rats fed diets containing 120 ppm zinc (9) had lower body weights and liver copper concentrations than those of the present study. The data presented here clearly demonstrate that rats fed the copper-deficient diets were indeed copper depleted. Serum ceruloplasmin activity, hepatic and splenic copper contents were all lowered in the copper deficient rats than in controls. However, the copper deficiency was not a severe one characterized by anemia, cardiomegaly, and coarse fur. In the present experiment the levels of minerals in the diet were specifically chosen at the NRC suggested level. Under these conditions when zinc is fed at the level suggested by the NRC, hypercholesterolemia was not associated with copper deficiency.

Although iron and copper deficiencies each produce alterations in the levels of the other mineral in organs, there were no detectable effects of this interaction on serum lipid concentrations. The effects of dietary iron and copper on the metabolism of lipids appear to be independent.

ACKNOWLEDGEMENTS

This investigation was supported by University of Illinois Biomedical Research grant No. RR07030 and by Hatch project No. 60-337, University of Illinois Agricultural Experiment Station. The technical assistance of Karen Sprehe Ruckman, Peggy Moran, and Lisa Steffensen is appreciated.

REFERENCES

1. GUTHRIE, H.A., FROOZANI, M., SHERMAN, A.R. and BARRON, G.P. Hyperlipidemia in offspring of iron-deficient rats. J. Nutr. 104: 1273-1278, 1974.

2. AMINE, E.K., DESILET, E.J. and HEGSTED, D.M. Effect of dietary fats on lipogenesis in iron deficiency anemic chicks and rats. J. Nutr. 106: 405-411, 1976.

3. AMINE, E.K. and HEGSTED, D.M. Iron deficiency lipemia in the rat and chick. J. Nutr. 101: 1575-1582, 1971.

4. LEWIS, M. and IAMMARINO, R.M. Lipemia in rodent iron deficiency. J. Lab. Clin. Med. 78: 547-554, 1971.

5. SHERMAN, A.R., GUTHRIE, H.A., WOLINSKY, I. and ZULAK, I.M. Iron deficiency hyperlipidemia in 18-day-old rat pups: Effects of milk lipids, lipoprotein lipase, and triglyceride synthesis. J. Nutr. 108: 152-162, 1978.

6. SHERMAN, A.R., GUTHRIE, H.A. and WOLINSKY, I. Interrelationships between dietary iron and tissue zinc and copper levels and serum lipids. Proc. Soc. Exper. Biol. Med. 156: 396-401, 1977.

7. FRIEDEN, E. and HUIEH, A.S. Ceruloplasmin: The copper transport protein with essential oxidase activity. Adv. Enzymol. 44: 187-236, 1976.

8. ALLEN, K.G.D. and KLEVAY, L.M. Copper deficiency and cholesterol metabolism in the rat. Atherosclerosis 31: 259-271, 1978.

9. LEI, K.Y. Cholesterol metabolism in copper-deficient rats. Nutr. Rept. Intl. 15: 597-605, 1977.

10. LEI, K.Y. Oxidation, excretion, and tissue distribution of [26-^{14}C] cholesterol in copper-deficient rats. J. Nutr. 108: 232-237, 1978.

11. PETERING, H.G., MURTHY, L. and O'FLAHERTY, E. Influence of copper and zinc on rat lipid metabolism. Agr. and Food Chemistry 25: 1105-1109, 1977.

12. SHAO, M.J.S. and LEI, K.Y. Conversion of [2-^{14}C] mevolonate into cholesterol, lanosterol, and squalene in copper-deficient rats. J Nutr. 110: 859-867, 1980.

13. ALLEN, K.G.D. and KLEVAY, L.M. Hyperlipidemia in rats due to copper deficiency. Nutr. Rept. Int. 22: 295-299, 1980.

14. SHERMAN, A.R. Lipogenesis in iron-deficient adult rats. Lipids 13: 473-478, 1978.

15. SHERMAN, A.R. and TSCHIEMBER, N.T. Tissue iron, copper, and zinc levels in offspring of iron-sufficient and iron-deficient rats. J. Nutr. 111: 266-275, 1981.

16. SHERMAN, A.R. Serum lipids in suckling and post-weanling iron-deficient rats. Lipids 14: 888-892, 1979.

17. HART, E.B., STEENBOCK, H., WADDELL, D. and ELVEHJEM, C.A. Iron in nutrition. VII. Copper as a supplement to iron for hemoglobin building in the rat. J. Biol. Chem. 77: 797-812, 1928.

18. EVANS, J.L. and ABRAHAM, P.A. Anemia, iron storage and ceruloplasmin in copper nutrition in the growing rat. J. Nutr. 103: 196-201, 1973.

19. OSAKI, S., JOHNSON, D.A. and FRIEDEN, E. The mobilization of iron from perfused mammalian liver by a serum copper enzyme, ferroxidase I. J. Biol. Chem. 249: 3018-3023, 1971.

20. RAGAN, H.A., NACHT, S., LEE, G.R., BISHOP, C.R. and CARTWRIGHT, G.E. Effect of ceruloplasmin on plasma iron in copper-deficient swine. Am. J. Physiol. 217: 1320-1323, 1969.

21. ROESER, H.P., LEE, G.R., NACHT, S. and CARTWRIGHT, G.E. The role of ceruloplasmin in iron metabolism. J. Clin. Invest. 49: 2408-2417, 1970.

22. SOURKES, T.L., LOYD, K. and BIRNBAIM, H. Inverse relationship of hepatic copper and iron concentrations in rats fed deficient diets. Can. J. Bioch. 46: 267-271, 1968.

23. OWEN, C.A. Effects of iron on copper metabolism and copper on iron metabolism in rats. Am. J. Physiol. 224: 514-518, 1973.

24. GREGORIADIS, G. and SOURKES, T.L. Intracellular distribution of copper in the liver of the rat. Can. J. Biochem. 45: 1841-1851, 1967.

25. NATIONAL RESEARCH COUNCIL. Nutritional requirements of laboratory animals. National Research Council, Washington, D.C., 1978.

26. RICHTERICH, R. Clinical Chemistry. Academic Press, NY, 1969.

27. HOUCHIN, O.B. A rapid colorimetric method for the quantitative determination of copper oxidase activity (ceruloplasmin). Clin. Chem. 4: 519-523, 1958.

28. FOLCH, J., LEES, M. and SLOANE STANLEY, G.H. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226: 497-509, 1957.

29. STERN, I. and SHAPIRO, B. A rapid and simple method for the determination of esterified fatty acids and for total fatty acids in blood. J. Clin. Path. 6: 158-160, 1953.

30. SEARCY, R.L. and BERGQUIST, L.M. A new color reaction for the quantitation of serum cholesterol. Clin. Chin. Acta 5: 192-199, 1960.

31. STEEL, R.G. and TORRIE, J.H. Principles and procedures of statistics. McGraw-Hill, NY, 1960.

32. HILL, C.H. and MATRONE, G. Chemical parameters in the study of in vivo and in utro interactions of transition elements. Fed. Proc. 29: 1474-1481, 1970.

NUTRITION RESEARCH, Vol. 1, pg. 373-384, 1981
0271-5317/81/040373-12$02.00/0 Printed in the USA.
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EFFECT OF MALNUTRITION AND BCG VACCINATION ON MACROPHAGE ACTIVATION IN GUINEA PIGS[1,2]

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ABSTRACT

Host defense functions, particularly cell-mediated immunity, may be profoundly altered by malnutrition. Activated macrophages are required as effector cells in the final stages of a successful cell-mediated immune response against microbial pathogens. Macrophage activation was assessed by measuring the activity of superoxide dismutase (SOD) in peritoneal macrophages obtained from BCG-vaccinated and non-vaccinated guinea pigs maintained on purified diets containing 8% or 25% casein. Protein deficiency, as measured by reduced hemoglobin and serum albumin concentrations, was accompanied by significant reductions in delayed hypersensitivity to purified protein derivative (PPD) and impaired lymphocyte blastogenic responses in vitro to PPD and phytohemagglutinin. In contrast, macrophage SOD activity was inversely correlated with protein nutritional status, irrespective of vaccination. These results suggest that the thymus-dependent (T) lymphocyte subsets responsible for delayed hypersensitivity, blastogenesis and macrophage activation may be affected differently by protein deprivation. Alternatively, enhanced macrophage activity may not be the result of T cell function in this model.

KEY WORDS: Protein malnutrition, cell-mediated immunity, macrophage activation, superoxide dismutase

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[1] Presented, in part, at the Federation of American Societies for Experimental Biology, Atlanta, GA, April, 1981.

[2] Portions of this study were submitted in partial fulfillment of the requirements for a Master of Science degree (T. Burch) at Texas A&M University.

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INTRODUCTION

Macrophages, and their blood monocyte precursors, are absolutely required as effector cells in the final stages of a successful cell-mediated immune response against certain microbial pathogens (1,2). Macrophage activity is regulated by thymus-dependent (T) lymphocytes through lymphokines produced as a result of a specific immunologic stimulus (2,3). Activated macrophages exhibit enhanced bactericidal and tumoricidal properties, concomitant with changes in the levels of several intracellular enzymes (3,4,5).

Protein-calorie malnutrition (PCM) is associated with profound alterations of host defense function. In humans, both severe and moderate malnutrition cause impaired T-lymphocyte function as measured by delayed hypersensitivity responses and lymphocyte blastogenesis in vitro (6,7,8). The bactericidal capacity of blood monocytes is reduced in children with kwashiorkor (9). Experimental animal studies have produced conflicting results. Cell-mediated immunity is enhanced by moderate protein deprivation in some model systems (10,11), while others have reported T-lymphocyte dysfunction similar to that observed in humans (12). Phagocytic and microbicidal activity was impaired in some studies of macrophages from protein-deficient animals (13,14), while others have demonstrated normal or enhanced enzymatic and phagocytic activity in malnourished animals (10,15,16).

Superoxide dismutase (SOD-E.C.1.15.1.1) is a macrophage-associated enzyme which protects the phagocytizing cell from superoxide anion generated during phagocytosis (17,18). Previous studies suggest that SOD may be an important enzyme in the successful encounter between macrophage and microbe (4,5). Watson el al. demonstrated increased SOD activity in alveolar macrophages from protein-deficient rats and guinea pigs and postulated that macrophage activation by enhanced T lymphocyte activity in protein-malnourished animals may explain their observations (15). Unfortunately, concomitant measures of cell-mediated immune function were not made in that study. Previously, we have described a model of PCM in guinea pigs which is characterized by impaired T lymphocyte function (12). Using SOD activity as an indicator of macrophage activation, we examined the effect of moderate protein deficiency on macrophage function and cell-mediated immunity in BCG-vaccinated and non-vaccinated guinea pigs.

MATERIALS AND METHODS

Experimental animals

Outbred, albino, specific pathogen-free female guinea pigs, weighing 200-300 gr, were obtained from a commercial source (Hartley-COBS, Charles River, Wilmington, MA). They were housed individually in polycarbonate cages on stainless wire mesh floors and provided with tap water and food ad libitum. Each animal was randomly assigned to an experimental treatment. Body weights were recorded twice weekly during the experiment.

Experimental diets

The experimental diets were isocaloric, with the casein and cornstarch content varying inversely to provide protein content of 8% or 25% casein. The formulations of the basic diet, mineral mix and vitamin mix have been published previously (12). The casein was supplemented with arginine and

methionine in the dietary group receiving 25% casein. The diets were prepared with agar gel, according to Navia and Lopez (19), and provided freshly every other day.

BCG vaccination

Half of the guinea pigs were vaccinated with viable bacille Calmette-Guerin (BCG) vaccine (Copenhagen 1331 - Statens Seruminstitut, Copenhagen, Denmark). Each animal received 0.1 ml saline containing about 1×10^3 viable bacilli subcutaneously in the left inguinal area. Non-vaccinated animals received the same amount of saline alone. The viability of the vaccine was determined by plating on oleic acid albumin (OAA) agar (DIFCO, Detroit, MI).

PPD skin tests

Delayed hypersensitivity was elicited in BCG-vaccinated guinea pigs by the intradermal injection of 0.1 ml purified protein derivative (PPD-RT-23; Statens Seruminstitut, Copenhagen, Denmark). Two doses of PPD, 5 and 100 tuberculin units (TU), were injected in different sites on a shaved area of the side. The reactions were measured with a transparent plastic ruler at 48 hours and the mean diameter of induration used to calculate the area (mm^2) of the reaction.

Macrophage induction and autopsy

Six weeks following vaccination and initiation of the experimental diets, groups of guinea pigs were sacrificed by cervical dislocation. Seventy-two hours prior to sacrifice the animals were injected intraperitoneally with 10 ml of 1.2% sterile sodium caseinate (Sigma Chemical, St. Louis, MO) in normal saline to induce peritoneal macrophage accumulation (18). The animals were exsanguinated by cardiac puncture with a heparinized 10 ml syringe. The abdominal cavity was opened aseptically and washed three times with 50 ml aliquots of sterile 0.10 M phosphate buffered saline (PBS), pH 7.4.

Hemoglobin concentrations were determined using a standard clinical procedure. Total protein content of the serum was measured using the Lowry method (20). Protein electrophoresis was carried out on cellulose acetate strips, which were stained and quantified in a scanning densitometer (DCD-16, Gelman Sciences, Ann Arbor, MI). Serum albumin concentrations were calculated on that basis.

Lymphocyte blastogenesis

A modified whole blood culture technique was used (21). Venous blood was collected and mixed immediately with preservative-free sodium heparin (Sigma Chemical, St. Louis, MO) to a final concentration of 50 U/ml. The blood was diluted 1:15, 1:30 and 1:60 with Medium 199 containing 10% fetal calf serum and antibiotics to a final volume of 3 ml. Triplicate cultures were stimulated with either phytohemagglutinin (PHA-P, Difco, Detroit, MI) or purified protein derivative (PPD; NIH-Trudeau Institute; lyophilized) at concentrations previously determined to give maximum blastogenic responses in lymphocytes from normal guinea pigs. Control cultures received nothing. The tubes were tightly sealed and incubated for 3,4 or 5 days at 37°C. Twenty four hours prior to harvest, all cultures were labeled with tritiated thymidine (^{3}H-TdR; New England Nuclear, North Bellerica, MA) to give a final concentration of 0.8 μCi/ml. The procedure for harvesting the radio-labeled DNA has been described previously (22). Samples were counted in a liquid

scintillation counter (LS-8000; Beckman Instruments, Irvine, CA) and the counts corrected for background and quenching and expressed as counts per minutes (cpm) per 10^6 viable lymphocytes. The optimal response of each animal's cells was selected as the value to represent that animal in data analyses.

Macrophage separation and fractionation

The pooled peritoneal washings were centrifuged at 2,000 x g for 15 minutes at 4°C, washed three times with cold Krebs Ringer Phosphate Buffer (KRPB), pH 7.4 , counted and resuspended in sufficient KRPB to give 2.5 x 10^6 cells/ml.

Approximately 8.0 ml of the suspension were overlayed on 5.0 ml of Ficoll-Paque (Pharmacia, Piscataway, NJ) and centrifuged 45 minutes at 18°C at 400 x g. Cells at the interface were collected. This step separated the contaminating polymorphonuclear leukocytes from the macrophages. The cells then were washed three more times in KRPB.

The cells were disrupted at 4°C for one minute (Branson Sonifier, Branson Sonic Power Co., Danburg, CT), and the sonicates centrifuged at 16,000 x g for 20 minutes at 4°C. The resulting pellet was resuspended with 0.2% Triton X-100 (Sigma Chemical, St. Louis, MO) to lyse mitochondria, lysosomes, and other cell organelles. The 16,000 x g pellet and supernatant fluid were assayed for cyanide insensitive superoxide dismutase. The protein concentration of each fraction was determined by the Folin method using crystalline human albumin as a standard (20).

Superoxide dismutase assay

Superoxide dismutase activity was assayed according to McCord and Fridovich (23). This assay depends on the capacity of SOD to inhibit cytochrome C reduction mediated by the superoxide radical generated during the xanthine oxidase catalyzed oxidation of xanthine. The reaction mixture contained 0.15 M potassium phosphate buffer, pH 7.8, plus 0.3mM ethylenediamine- tetraacetic acid. Assays were conducted with and without 1.0 mM potassium cyanide. Xanthine (Sigma-grade 3) was incubated with xanthine oxidase (Sigma-grade 4) to generate the superoxide radical. The final concentrations were 50.0 µM xanthine and 0.01 units xanthine oxidase per 3.0 ml. To insure maximum sensitivity of the SOD, cytochrome C (Sigma-horse heart type VI) was added to the reaction mixture to a final concentration of 10.0 µM. To eliminate the effects of cytochrome oxidase, sodium azide was added to a final concentration of 1.0 mM. Superoxide dismutase (Sigma-Type I) was used as a standard. The change in optical density was read at 550 nm for two minutes with a Beckman DB recording spectrophotometer (Beckman Instr., Irvine, CA).

The total SOD activity was calculated by adding the total activity of the mitochondrial SOD and supernatant (cytosolic) SOD in the absence of potassium cyanide. The manganese SOD activity of each cell fraction was determined in the presence of potassium cyanide. The cooper-zinc SOD was calculated by subtracting the activity value of the enzyme in the presence of potassium cyanide from the total SOD in the appropriate cell fraction. The specific activity was obtained by dividing the SOD activity by the total protein content of each fraction (units/mg protein/min).

Statistical analysis

Throughout the experiment, principles of randomization were followed. Statistical Analysis System (SAS) (24), a computer package, was used to analyze the data. Multiple regression analysis was used to estimate the statistical coefficients in an interdependent system of linear equations. The analysis of variance was used to test the effect of diet on growth and serum protein status within vaccine group and when appropriate, Student's t-test was used to compare group means.

RESULTS

Nutritional status

The effects of consumption of purified diets containing 8% or 25% casein on growth, serum albumin and hemoglobin concentrations are illustrated in Fig. 1.

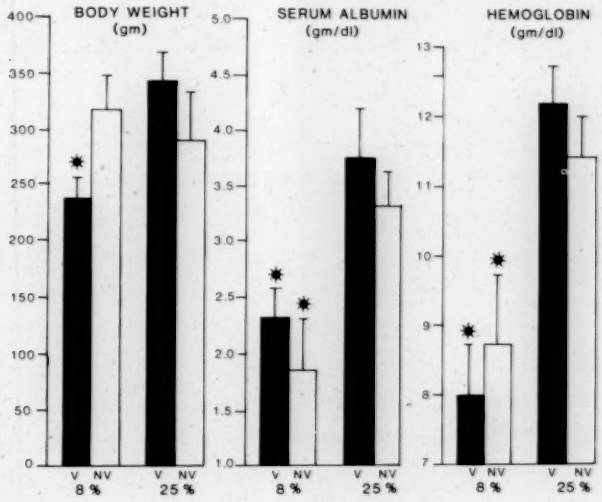


FIG. 1

Influence of dietary protein content on body weight, serum albumin and hemoglobin concentrations in BCG-vaccinated (■) and nonvaccinated (□) guinea pigs; mean + SEM: asterisk indicates significant difference between diet groups ($p < 0.05$).

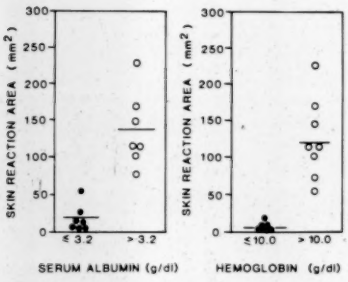
There was an influence of BCG vaccination on weight gain in the 8% casein group, with the vaccinated animals finishing the study significantly underweight. There was no effect of dietary protein content on the body weights of non-vaccinated guinea pigs. Animals on the low protein diet had significantly lower serum albumin concentrations, irrespective of vaccination, with most of the values under 2.5 gm/dl. Similarly, significant anemia was detected in protein-deprived animals as measured by dramatic reductions in hemoglobin concentrations (less than 10 gm/dl). Vaccination status had no effect on hemo-

globin levels.

Delayed hypersensitivity to PPD

The response of BCG-vaccinated guinea pigs to PPD skin tests was significantly impaired by protein deficiency (Fig. 2).

FIG 2.



Effect of protein status on delayed hypersensitivity reactions to 100 TU dose of PPD in BCG-vaccinated guinea pigs; reactions measured 48 hr. post-injection; horizontal bar indicates group mean.

Guinea pigs with serum albumin concentrations less than 3.2 gm/dl and hemoglobin levels less than 10 gm/dl were practically anergic to PPD. Guinea pigs with values above these levels responded with strong delayed hypersensitivity reactions with mean areas of induration between 120-140 mm^2.

Lymphocyte blastogenesis in vitro

Figure 3 illustrates the significant positive correlation which was observed between serum albumin concentrations and the response of peripheral blood lymphocytes to phytohemagglutinin (PHA) (Fig. 3A) and PPD (Fig. 3B).

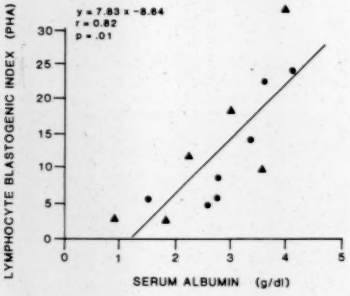


FIG. 3A

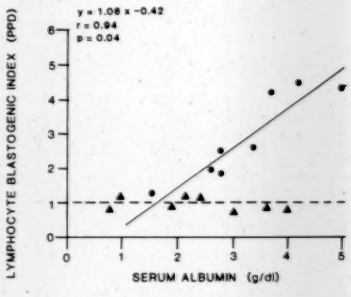


FIG. 3B

Relationship between protein status and in vitro blastogenic responses of peripheral blood lymphocytes in BCG-vaccinated (●) and non-vaccinated (▲) guinea pigs. Blastogenic Index is the ratio of cpm in stimulated vs unstimulated cultures.

Lymphocytes from protein deficient animals responded poorly to PHA stimulation and BCG vaccination had no effect on this relationship. As expected, lymphocytes from non-vaccinated guinea pigs were uniformly unresponsive to PPD in vitro, while the impairment associated protein deficiency was quite apparent in the BCG-vaccinated group.

Superoxide dismutase activity

A significant inverse correlation was observed between SOD activity in peritoneal exudate macrophages and the protein status of BCG-vaccinated and non-vaccinated guinea pigs (Fig. 4). Macrophages from protein-deficient animals had elevated total SOD activity (Fig. 4A), irrespective of vaccination status. Total mitochondrial SOD activity (Fig. 4B) was also significantly elevated ($p < 0.01$) and contributed almost entirely to the overall effect, since total supernatant SOD was not affected by protein status ($p < 0.17$). Within the mitochrondrial SOD activity, the manganese (cyanide insensitive) fraction, but not the copper-zinc fraction, was most influenced by albumin levels ($p < 0.05$).

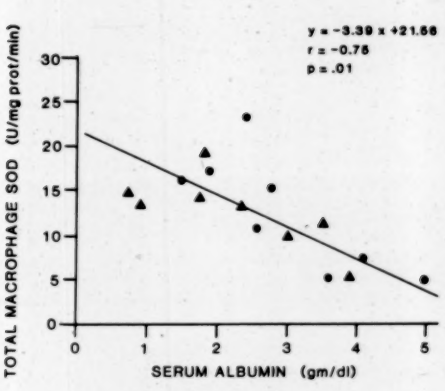


FIG. 4A

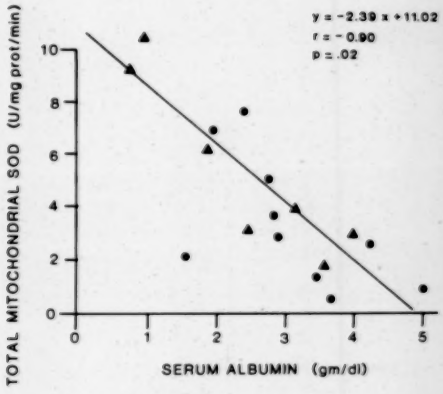


FIG. 4B

Relationship between serum albumin concentration and total (A) or total mitochondrial (B) superoxide dismutase (SOD) activity in peritoneal exudate macrophages from BCG-vaccinated (●) or nonvaccinated (▲) guinea pigs.

Similar results were obtained when total mitochondrial SOD activity was regressed on hemoglobin levels (Fig. 5). A significant inverse correlation ($p < 0.04$) was obtained, to which the copper-zinc fraction contributed almost entirely ($p < 0.02$). Again, supernatant SOD activity was unaffected by hemoglobin status. Vaccination did not affect the interaction between hemoglobin and SOD activity.

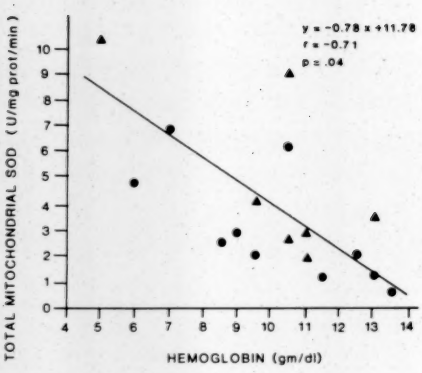


FIG. 5

Correlation between hemoglobin levels and total mitochondrial superoxide dismutase (SOD) activity in peritoneal exudate macrophages from BCG-vaccinated (●) and nonvaccinated (▲) guinea pigs.

DISCUSSION

We have demonstrated increased activation, as measured by superoxide dismutase (SOD) levels, of peritoneal exudate macrophages from protein-deficient guinea pigs. Enhanced macrophage activity was found to be independent of vaccination with viable BCG, and occurred in the face of impaired cell-mediated immune function in the malnourished animals. Tuberculin anergy and reduced lymphocyte blastogenesis *in vitro* in protein-deprived guinea pigs has been observed previously in this model (12). Our results suggest that enhanced macrophage activity in protein deficiency is not the result of amplified T lymphocyte function, as has been suggested by other workers (15).

These findings confirm and extend the report of Watson et al, who observed increased SOD activity in alveolar macrophages from protein-malnourished guinea pigs and rats (15). Others have reported enhanced macrophage function in experimental malnutrition, including increased phagocytosis of *Listeria monocytogenes* by peritoneal macrophages (10). Acute starvation protected against infection with *L. monocytogenes* in mice whose peritoneal macrophages inhibited DNA synthesis of tumor cells to a greater extent than well-fed controls (25). Kramer et al found that lymphocytes from guinea pigs maintained on 6-8% casein diets produced more of a lymphokine which inhibits macrophage migration (MIF) (11). Keusch and coworkers observed normal morphology, chemotaxis and bactericidal activity in peritoneal macrophages from protein-calorie malnourished rats (16,26). These observations contrast sharply with reports of impaired phagocyte function in malnutrition. Bhuyan and others demonstrated impaired macrophage accumulation and failure to form granulomas in protein-deficient guinea pigs and rabbits immunized with BCG (27,28). Vitamin C deficiency impaired the migration of macrophages from scorbutic guinea pigs, although phagocytic capacity was normal (29). In mice, phagocytic uptake of chicken erythrocytes (13) and macrophage-mediated resistance to Wesselsbron virus (14) were found to be compromised by dietary protein deficiencies. In the latter study, the macrophage virucidal defect was confirmed *in vitro* (14).

Others have demonstrated normal or enhanced T lymphocyte-mediated immune functions in moderately protein-deficient animals, specifically allograft

rejection, graft vs host reaction, and in vitro blastogenesis (10,11,30). Our results, in contrast, are analogous to those observed repeatedly in malnourished humans (6-8). Given the functional heterogeneity of T lymphocyte subsets (31), it seems reasonable to postulate a differential effect of protein-calorie malnutrition on subsets which mediate the various functions measured, thus explaining the discrepancies in the literature. There is some experimental evidence supporting such an hypothesis (32,33). In the present study, malnutrition was shown to impair delayed hypersensitivity reactions and blastogenic responses to a polyclonal T cell mitogen which may reflect T cell dysfunction. Alternatively, macrophage activation, as reflected by increased SOD activity, may be independent of T lymphocyte function under these experimental conditions.

Impaired PPD skin test responses in malnourished individuals vaccinated with BCG imply that the vaccine has not stimulated a protective immunity (34), although a number of non-immunologic factors may also influence the delayed hypersensitivity reaction. Our results demonstrate a direct effect of protein deprivation on the lymphocyte subset responsive to PPD (Fig. 3B), which may explain the tuberculin anergy observed in the low protein animals (Fig. 2). Thus, protein malnutrition appears to impair the immunologic mechanisms responsible for the development of cell-mediated immunity to tuberculoproteins, an observation which has profound implications for BCG vaccine efficacy in malnourished populations (35).

The serum albumin concentration has been shown clearly to be a sensitive indicator of protein nutritional status, which correlates well with other biochemical and functional indicators of malnutrition (36). The use of serum albumin, rather than dietary protein level, as the predictor variable in this study was predicated upon the variation in responses of individual animals to the experimental diets and the desire to establish albumin levels as a biochemical correlate of functional abnormalities in this experimental model. Our results confirm this relationship and provide a rationale for the use of serum albumin as an independent variable in future studies.

The significant reduction in weight gain observed in BCG-vaccinated, but not in non-vaccinated, guinea pigs fed the 8% casein diet is similar to the results of Kielmann et al in children receiving living vaccines (37). It appears that while the 8% diet resulted in measurable decreases in serum albumin and hemoglobin, body weight was not affected in the absence of additional stress such as that afforded by the BCG infection. In contrast, animals consuming an adequate diet were not adversely affected by the vaccination. These observations have obvious implications for the use of live vaccines in marginally nourished populations (37).

ACKNOWLEDGEMENTS

The authors thank Ms. Cheryl Barbe, Ms. Denise Luker and Ms. Deborah Armes for excellent technical assistance. Supported, in part, by NIH Grant No. AI-15495 and the American Lung Association.

NOTE

The research for this project was conducted by E. A. Yetley during her tenure at Texas A&M University. The opinions expressed represent those of the authors, not the policy or opinions of the Food and Drug Administration where Dr. Yetley currently is employed.

REFERENCES

1. MACKANESS, G. B. The immunological basis of acquired cellular resistance. J. Exp. Med. 120: 105-120, 1964.

2. FOWLES, R. E., FAJARDO, I. M., LEIBOWITCH, J. L. and DAVID, J. R. The enhancement of macrophage bacteriostatis by products of activated lymphocytes. J. Exp. Med. 138: 952-964, 1973.

3. SIMON, H. B. and SHEAGREN, J. N. Enhancement of macrophage bactericidal capacity by antigenically stimulated immune lymphocytes. Cell. Immunol. 4: 163-174, 1972.

4. JOHNSTON, R. B., JR., GODZIK, C. A. and COHN, Z. A. Increased superoxide anion production by immunologically activated and chemically elicited macrophages. J. Exp. Med. 148: 115-121, 1978.

5. JOHNSTON, R. B., KEELE, B. B., MISRA, H. P., LEHMEYER, J. E., WEBB, L. S., BAEHNER, R. L. and RAJAGOPALAN, K. V. The role of superoxide anion generation in phagocytic bactericidal activity. J. Clin. Invest. 55: 1357-1372, 1975.

6. McMURRAY, D. N., LOOMIS, S. A., CASAZZA, L. J., REY, H. and MIRANDA, R. Development of impaired cell-mediated immunity in mild and moderate malnutrition. Am. J. Clin. Nutr. 34: 68-77.

7. ZIEGLER, H. D. and ZIEGLER, P. B. Depression of tuberculin reaction in mild and moderate protein-calorie malnourished children following BCG vaccination. Johns Hopkins Med. J. 137: 59-64, 1975.

8. NEUMANN, C. G., LAWLOR, G. J., STIEHM, E. R., SWENDSEID, M. E., NEWTON, C., HERBERT, J., AMMANN, A. J. and JACOB, M. Immunologic responses in malnourished children. Am. J. Clin. Nutr. 28: 89-104, 1975.

9. DOUGLAS, S. D. and SCHOPFER, K. Phagocyte function in protein-calorie malnutrition. Clin. Exp. Immunol. 17: 121-128, 1974.

10. COOPER, W. C., GOOD, R. A. and MARIANI, T. N. Effect of protein insufficiency on immune responses. Am. J. Clin. Nutr. 27: 647-664, 1974.

11. KRAMER, T. R. and GOOD, R. A. Increased in vitro cell-mediated immunity in protein malnourished guinea pigs. Clin. Immunol. Immunopathol. 11: 212-228. 1978.

12. McMURRAY, D. N. and YETLEY, E. A. Immune responses in malnourished guinea pigs. J. Nutr. (submitted).

13. HOOK, R. R., JR., HUTCHESON, D. P. and WOODWARD, K. Influence of protein-calorie malnutrition on in vivo phagocytosis by peritoneal macrophages of C57BL/6J mice. Nutr. Rep. Intl. 9: 191-196, 1974.

14. OLSON, L. C., SISK, D. R. and IZSAK, E. Protein-calorie malnutrition impairs the antiviral function of macrophages. Proc. Soc. Exp. Biol. Med. 159: 84-87, 1978.

15. WATSON, R. R., RISTER, M. and BAEHNER , R. L. Superoxide dismutase activity in polymorphonuclear leukocytes and alveolar macrophages of protein malnourished rats and guinea pigs. J. Nutr. 106: 1801-1808, 1976.

16. KEUSCH, G. T., DOUGLAS, S. D., HAMMER, G. and BRADEN, K. Antibacterial functions of macrophages in experimental protein-calorie malnutrition. II. Cellular and humoral factors for chemotaxis, phagocytosis and intracellular bactericidal activity. J. Infect. Dis. 138: 134-142, 1978.

17. DRATH, D. B. and KARNOVSKY, M. L. Superoxide production by phagocytic leukocytes. J. Exp. Med. 141: 257-263, 1975.

18. RISTER, M. and BAEHNER , R. L. A comparative study of superoxide dismutase activity in polymorphonuclear leukocytes, monocytes and alveolar macrophages of the guinea pig. J. Cell. Physiol. 87: 345-351, 1976.

19. NAVIA, J. and LOPEZ, H. A purified agar-gel diet for guinea pigs. Lab Anim. Sci. 23: 111-113, 1973.

20. LOWRY, O. H., ROSENBROUGH, N. J., FARR, A. L. and RANDALL, R. J. Protein measurements with the Folin-phenol reagent. J. Biol. Chem. 193: 265-275, 1951.

21. HAN, T. and PAULY, J. Simplified whole blood method for evaluating in vitro lymphocyte reactivity of laboratory animals. Clin. Exp. Immunol. 11: 137-142, 1972.

22. JUNGE, U., HOEKSTRA, J., WOLFE, L. and DEINHARDT, F. Microtechnique for quantitative evaluation of in vitro lymphocyte transformation. Clin. Exp. Immunol. 7: 431-437, 1970.

23. McCORD, J. M. and FRIDOVICH , I. Superoxide dismutase: an enzymic function for erythrocuprein (hemocuprein). J. Biol. Chem. 244: 6049-6055, 1969.

24. BARR, A. H., GOODNIGHT, J. H., SALL, J. P. and HELWIG, J. T. A User's Guide to SAS 76, SAS Institute, Inc., Raleigh, N. C., 1976.

25. WING, E. J. and YOUNG, J. B. Acute starvation protects mice against Listeria monocytogenes. Infect. Immun. 28: 771-776, 1980.

26. KEUSCH, G. T., DOUGLAS, S. D., BRADEN, K. and GELLER, S. A. Antibacterial functions of macrophages in experimental protein-calorie malnutrition. I. Description of the model, morphologic observations, and macrophage surface IgG receptors. J. Infect. Dis. 138: 125-133, 1978.

27. BHUYAN, U. N. and RAMALINGASWAMI,V. Immune responses of the protein-deficient guinea pig to BCG vaccination. Am. J. Pathol. 72: 489-502, 1973.

28. BHUYAN, U. N. and RAMALINGASWAMI, V. Systemic macrophage mobilization and granulomatous response to BCG in the protein-deficient rabbit. Am. J. Pathol. 76: 313-322, 1974.

29. GANGULY, R., DURIEUX, M.-F. and WALDMAN, R. H. Macrophage function in vitamin C-deficient guinea pigs. Am. J. Clin. Nutr. 29: 762-765, 1976.

30. GOOD, R. A., FERNANDES, G., YUNIS, E. J., COOPER, W. C., JOSE, D. C., KRAMER, T. R. and HANSEN, M. A. Nutritional deficiency, immunologic function, and disease. Am. J. Pathol. 84: 599-614, 1976.

31. CANTOR, H. and BOYSE, E. A. Regulation of cellular and humoral immune responses by T-cell subclasses. Cold Spring Harbor Symp. Quant. Biol. 41: 23-32, 1976.

32. CHANDRA, R. K. Lymphocyte subpopulations in human malnutrition: cytotoxic and suppressor cells. Pediatrics 59: 423-427, 1977.

33. CHANDRA, R. K. T and B lymphocyte subpopulations and leukocyte terminal deoxynucleotidyl-transferase in energy-protein undernutrition. Acta Paediatr. Scand. 68: 841-845, 1979.

34. KNIGHT-SHAPIRO, C. D., HARDING, G. E. and SMITH, D. W. Relationship of delayed hypersensitivity and acquired cellular resistance in experimental airborne tuberculosis. J. Infect. Dis. 130: 8-15, 1974.

35. Tuberculosis Prevention Trial, Madras. Trial of BCG vaccines in South India for tuberculosis prevention. Ind. J. Med. Res. 70: 349-363, 1979.

36. HAY, R. W., WHITEHEAD, R. G. and SPICER, C. C. Serum-albumin as a prognostic indicator in oedematous malnutrition. Lancet 2: 427, 1975.

37. KIELMANN, A. A. Weight fluctuations after immunization in a rural preschool child community. Am. J. Clin. Nutr. 30: 592-602, 1977.

NUTRITION RESEARCH, Vol. 1, pg. 385-392, 1981
0271-5317/81/040385-08$02.00/0 Printed in the USA.
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PRENATAL PROTEIN-ENERGY MALNUTRITION AND ITS RECOVERY OF MANDIBLE AND LONG BONE IN NEWBORN RATS

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ABSTRACT

Pregnant rats were fed either a 25% protein diet as controls or a 10% protein diet from day 13 of gestation to birth. Immediately after birth (day 1), calcium uptake and collagen synthesis were studied in the mandible and long bone of the newborns. Control dams were fed continuously a 25% protein diet and prenatally malnourished dams were fed either a 25% protein diet or continuously a 10% protein diet until day 15. The mandibles and long bones were then studied to determine the recovery of the prenatal malnutrition at day 15. At the time of birth, body weight and the weights of the mandibles and long bones were affected to approximately the same extent. Calcium and collagen contents of the mandible in the malnourished groups were less when compared to the controls, but no differences were observed in the long bone between groups. On the other hand, calcium uptake and collagen synthesis in the mandibles were greater in the malnourished group than that of the controls, but no differences in the long bones were observed. At day 15, body weight and the weights of the mandibles and long bones in the rehabilitated group were about the same as that of the controls. Furthermore, there was no difference in calcium and collagen contents as well as collagen synthesis between these groups, but calcium uptake in the rehabilitated group was greater. The differences in body weights and the weights of the mandibles and long bones of the continuously malnourished group compared to the controls were increased by day 15. The calcium and collagen contents of this group were less, also. However, calcium uptake and collagen synthesis in the mandibles and long bones in the continuously malnourished group were increased compared to the control group. It is concluded that both mandibles and long bones can be successfully rehabilitated, if refeedings are initiated properly and early.

KEY WORDS: Protein-energy malnutrition, rehabilitation, mandible, long bone, newborn rat.

The effects of protein-energy malnutrition during pregnancy and the early prenatal period have been well documented (1-3). In pigs and rhesus monkeys suffering from protein-energy malnutrition, the skull and long bones are smaller with reduced osteoblastic activity (4,5). Recent studies (6) have shown that prenatally malnourished offspring also demonstrate growth reduction of the mandibles and long bones. Although rehabilitation following prenatal malnutrition has been studied in brain (7), muscle (8) and adipose tissue (9), very little attention has been given to that of the hard tissues following early malnutrition. It seems that the timing of refeeding is, in general, extremely important in determining whether one can recovery to the full extent from the malnourished state in the later stages of life. A recent study showed that relatively large numbers of pregnant women were not adequate in their protein intake (10). Offspring born from such nutritionally deprived mothers seem also affected (11). The present study was conducted to reveal whether or not the bones of the offspring whose mothers were protein-energy malnourished prenatally can recover by having their mother adhere to an adequate protein diet postnatally.

MATERIALS AND METHODS

A total of 16 pregnant rats (Holtzman strain, Madison, WI) were used. Six control dams were fed, ad libitum, a 25% protein diet from the 13th day of gestation until birth. The other 10 dams were similarly fed an isoenergetic diet, but provided only 10% protein (Table 1). Within 8 hours of delivery from the dams, randomly picked pups were weighed and designated as day 1. Since there was no difference in the effect of malnutrition attributable to sex in early ages (12), sex was not differentiated. All pups from the control dams were gathered, and then randomly reassigned to dams within that group, eight pups to each dam. This process was repeated for the malnourished group. At this time calcium uptake and collagen synthesis studies were conducted on some of these pups to determine day 1 values. A total of 16 pups in the control and 16 in the malnourished group were used.

Four control dams were provided with the same 25% protein diet (group 1). Four dams who were malnourished prenatally, were changed to a 25% protein diet upon birth (group 2). Four dams who were malnourished prenatally, were continuously provided with the same 10% protein diet (group 3). The body weights of the pups were measured at birth and every third day until 15. The pups were killed by cutting the carotid artery, and the mandibles and long bones were obtained as described (12).

TABLE 1

Composition of diet (g)

	25% Casein	10% Casein
Casein	250	100
Dextrose	172	252
Sucrose	168	162
Dextrin	172	248
Mazola Corn Oil	150 ml	150 ml
Mineral Mix*	40	40
Cholin Chloride (50% w/v)	4 ml	4 ml
Cellulose	35	35
Vitamin mix**	10	10
Methionine	----	1

*Rogers-Harper Mineral Mix (Teklad Test Diets, Madison, WI).
**AIN Vitamin Mixture 76 (ICN Pharmaceuticals, Inc., Cleveland, OH).

The mandible and the long bone were also weighed at birth and at day 15. Pups of each group were injected with ^{45}Ca (New England Nuclear, Boston, MA) at the dose of 100 µCi/100 gm. At day 1 and day 15, pups were killed one and one-half hours after injection to determine calcium uptake as described (13). The calcium content of these samples was measured by atomic absorption spectrophotometry (Model 280, Fisher Scientific Co., Fairlawn, N.J.). ^{45}Ca contents of the bone were then counted in a liquid scintillation spectrometer (Beckman Model LS - 3145T, Irvine, CA).

Other groups of pups at day 1 and 15 were injected with uniformly labeled ^{14}C proline (New England Nuclear, Boston, MA) at the dose of 6.5 µCi/100 g. Six hours later, they were killed, blood was collected, and the mandibles and long bones were obtained. Collagen synthesis was determined by the method described previously (13). Data were analyzed by the student's t-test (14).

RESULTS

At the time of birth, prenatally malnourished pups were smaller compared to those of the controls ($P < 0.05$). The differences of pups compared to the controls was increased when dams were kept on the same 10% protein diet until day 15 (group 3). At day 15, the body weight of continuously malnourished pups (group 3) was about one-half of that of the controls (group 1) ($P < 0.001$). On the other hand, the weights of the prenatally malnourished pups were recovered completely when mothers were fed a 25% protein diet postnatally (group 2). The postnatal body growth of these pups was almost identical to that of the control group ($P > 0.05$) (Table 2). The weights of the

mandibles and long bones in the prenatally malnourished group were also smaller compared with that of the controls at the time of birth ($P < 0.01$; $P < 0.05$). However,

TABLE 2

Changes of body weight from birth to day 15 (g)

	Days					
	1	4	7	10	13	15
Group 1	6.9[1,*] ±0.6	10.7** ±0.9	17.5*** ±1.4	25.0*** ±1.9	33.4*** ±2.4	38.6*** ±2.8
Group 2	6.2[2] ±0.6	9.5 ±1.5	16.7 ±2.7	24.5 ±2.9	33.3 ±3.2	39.2 ±3.7
Group 3		7.8 ±1.3	10.7 ±1.6	13.8 ±1.9	17.4 ±2.4	19.8 ±2.6

Group 1, A 25% protein diet was fed to dams from day 13 of gestation until birth. Upon delivery, a 25% protein diet was continuously fed to dams until day 15 postnatally.

Group 2, A 10% protein diet was fed to dams from day 13 of gestation until birth. Upon delivery, a 25% protein diet was fed to dams until day 15 postnatally (recovery group).

Group 3, A 10% protein diet was fed to dams from day 13 of gestation until birth. Upon delivery, a 10% protein diet was continuously fed to dams until day 15, postnatally.

[1]Mean ± S.D., A comparison between group 1 and 3 is; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; [2]Day 1, prenatally malnourished offspring were put together and weighed then some were killed. The rest of the offspring were randomly assigned to dams. Following this procedure dams were divided to Group 2 and 3.

at day 15, the differences in weight had disappeared between group 1 and 2 ($P > 0.05$). The differences in the weight of these bones in group 3 increased when compared to those of group 1 ($P < 0.001$) (Table 3).

TABLE 3

Average Weight of Mandible and Long Bone (mg)[1]

	Mandible	Long Bone	Mandible	Long Bone
Group 1	25.7[2*] ±2.8	19.7* ±2.0	93.8** ±6.0	184.5** ±16.5
Group 2	22.0 ±3.2	17.5 ±2.5	88.5 ±9.6	184.0 ±23.6
Group 3			61.1 ±11.7	107.1 ± 9.3

1 Wet weight compared to semi-wet weight previously reported (12)
2 Mean ± S.D.
A comparison between control and malnourished group at day 1, * $P < 0.05$ and between group 1 and 3 at day 15, ** $P < 0.001$.

At the time of birth calcium contents of the mandible from the prenatally malnourished pups were less than that of the controls ($P < 0.05$), but that of the long bone showed no difference ($P > 0.05$). However, at day 15, calcium contents of both mandibles and long bones in group 3 were much less than those of the controls (group 1)

($P < 0.01$ and $P < 0.001$). There was no difference between group 1 and 2 at day 15 ($P > 0.05$) (Table 4).

There was an increase of ^{45}Ca uptake in the mandibles of the prenatally malnourished pups compared to that of the controls at day 1 ($P < 0.05$), but that of the long bone was not different ($P > 0.05$). At day 15, both a mandible and long bone in group 2 showed higher ^{45}Ca uptake when compared to those of the controls (group 1) ($P < 0.001$). A comparison between group 1 and 3 indicated that there was even greater calcium uptake in the continuously malnourished group ($P < 0.001$) (Table 4).

Although there was a slight decrease of collagen contents in the mandible in the prenatally malnourished pups compared to that of the controls at day 1 ($P < 0.05$), no difference was observed in the long bone between these groups ($P > 0.05$). At day 15, collagen contents of both mandible and long bone in group 2 were not different compared to the controls. Collagen contents of both the mandible and long bone in group 3 were less than that of the controls ($P < 0.01$). At the time of birth, collagen synthesis in the mandible of the prenatally malnourished group was greater than that of the well-nourished group ($P < 0.005$) (Table 5).

Increased collagen synthesis in the mandible of this group occurred during the incorporation of blood proline into bone matrix ($P < 0.05$). In contrast, the long bone of the prenatally malnourished group was not different than that of the controls ($P > 0.05$). At day 15, there was no difference of collagen synthesis in both a mandible and long bone between group 1 and 2. However, collagen synthesis of continuously

TABLE 4

Calcium content and ^{45}Ca uptake per mandible and long bone

		Day 1		Day 15	
		Mandible	Long Bone	Mandible	Long Bone
Group 1	Ca content (mg)	1.4[1,*] ±0.1	0.4 ±0.1	17.7** ±2.3	5.9*** ±0.7
	DPM/Ca(mg) (x10⁴)	38.9 ±7.5	40.0 ±5.4	3.1 ±0.4	4.8 ±0.6
Group 2	Ca content (mg)	1.1[2] ±0.3	0.5 ±0.1	18.1 ±1.5	5.9 ±0.8
	DPM/Ca(mg) (x10⁴)	50.3[2]* ±12.4	37.9 ±4.6	18.2[a] ±1.9	29.1[a] ±6.6
Group 3	Ca content (mg)	1.1 ±0.3	0.5 ±0.1	5.4 ±0.7	2.8 ±0.6
	DPM/Ca(mg) (x10⁴)	50.3* ±12.4	37.9 ±4.6	34.3*** ±5.1	65.1*** ±10.7

1 Mean ± S.D. 2 Group 2 and 3 at day 1 are the same value as explained in the text

A comparison between control and malnourished group at day 1, * $P < 0.05$
A comparison between group 1 and 3 at day 15, ** $P < 0.01$, *** $P < 0.001$
Group 2 is different from group 1 and 3 at day 15, a $P < 0.001$

malnourished group in both the mandible and long bone was greater when compared to that of the controls ($P < 0.001$). Increased collagen synthesis in the mandible and long bone of this group occurred during the incorporation of blood proline into bone matrix ($P < 0.001$) (Table 5).

DISCUSSION

Protein-energy malnutrition during pregnancy induces many adverse effects on the soft tissue of the offspring (1-3). Hard tissues are not excepted from these effects

as indicated by the previous study (6). In the beginning of the studies, the group of pregnant dams were fed a 6% protein diet and changed to a 25% protein diet upon birth to study the recovery of these offspring. However, the pups born from a 6% protein diet which was fed to dams during pregnancy were too weak to suck continuously the dam's milk during a postnatal period. The mortality was extremely high during the early postnatal period, therefore, in the present study, dams were fed a 10% protein diet.

Although, at the time of birth, the body weights of the pups born from the dams fed a 10% protein diet were about 10% smaller compared to that of a 25% protein diet, the subsequent weight gain was approximately the same when dams were placed on a 25% protein diet postnatally. The body weights of the pre- and postnatally protein

TABLE 5

Collagen content and collagen synthesis in mandible and long bone

		Day 1		Day 15	
		Mandible	Long Bone	Mandible	Long Bone
Group 1	Total Collagen content(μmole)	0.61[2]* ±0.15	0.28 ±0.05	4.73* ±0.44	7.11* ±1.12
R.S.A.[1]	I	0.575 ±0.039	0.433 ±0.074	0.429 ±0.170	0.227 ±0.020
	II	0.443 ±0.021	0.466 ±0.012	0.710 ±0.079	0.559 ±0.097
	III	0.491 ±0.222	0.932 ±0.181	0.510 ±0.222	0.377 ±0.012
Group 2	Total Collagen content(μmole)	0.45[3] ±0.15	0.25 ±0.11	4.57 ±0.47	6.47 ±1.18
R.S.A.	I	0.704[3]** ±0.056	0.508 ±0.057	0.366 ±0.052	0.196 ±0.022
	II	0.551[3] ±0.192	0.593 ±0.130	0.665 ±0.234	0.545 ±0.039
	III	0.728[3]* ±0.034	0.875 ±0.103	0.674 ±0.111	0.366 ±0.019
Group 3	Total Collagen content(μmole)	0.45 ±0.15	0.25 ±0.11	3.44 ±0.24	4.62 ±0.62
R.S.A.	I	0.704** ±0.056	0.508 ±0.057	0.719** ±0.056	0.372** ±0.053
	II	0.551 ±0.192	0.593 ±0.130	0.644 ±0.194	0.553 ±0.123
	III	0.728* ±0.038	0.875 ±0.103	1.184** ±0.314	0.621** ±0.141

1. R.S.A.=Relative specific activity. This was calculated to provide an index of the uptake and/or metabolic conversion of proline by the tissue under consideration relative to that in the precursor pool as described (15). I-Bone hydroxyproline S.A./blood proline S.A. II-Bone hydroxyproline S.A./bone proline S.A. III-Bone proline S.A./blood proline S.A. 2. Mean ± S.D. 3. Group 2 and 3 at day 1 are the same value as explained in the text. A comparison between control and malnourished group at day 1. *$P<0.05$ **$P<0.005$. A comparison between group 1 and 3 at day 15. *$P<0.01$ **$P<0.001$.

malnourished offspring seem to return to the normal level, even if the proper nutritional refeeding is initiated a few weeks after birth (7). The body weights of young rats who are protein-energy malnourished have also been reported to be rehabilitated in both male and female rats (15).

The weights of the mandible and long bone from the prenatally malnourished pups at the time of birth was 14% and 11% less, respectively, when compared to that of the well nourished group, suggesting that the mandible and long bone were affected prenatally about the same extent as the total body weight. This contrasts to the postnatal malnutrition, where the mandible is less affected than the weights of total body and long bone (12). These bones were well recovered by day 15. On the other hand, the body weight of the continuously malnourished offspring was about 49% of that of the controls by day 15, but the weights of the mandible and long bone were about 35% and 42% less, suggesting mandibles were especially resistant to the nutritional stress compared to the effects of the total body weight. Furthermore, this suggests that the postnatal effects on the mandible and long bone were greater than that of the prenatal effects. The greater resistance of the mandible to the postnatal nutritional stress compared to that of the long bone has been well described (12,16).

At the time of birth, collagen contents of the mandible in the prenatally malnourished offspring were less when compared to that of the controls. This may account for the decreased weight of the mandible in the prenatally malnourished offspring, in parallel with the decreased calcium contents of this group. On the other hand, both collagen and calcium contents in the long bone in this malnourished group at day 1 were the same compared to the controls, regardless of the decreased weight in the malnourished group, suggesting that noncollagenous components might play some roles in the discrepancy of this weight.

It has been reported that the different steps of collagen synthesis of the mandible and long bone are affected in the postnatal protein-energy malnourished rats (13). In the present study, the increased incorporation of blood proline to bone matrix was observed when compared to the controls. This contrasts with the fact that no effects were observed in this step of long bone at day 1.

Increased collagen synthesis in parallel to the increased calcium uptake in the mandible of the prenatally malnourished group at day 1 contrasted with that of the long bone, where neither collagen synthesis nor calcium uptake of this group showed any differences compared to that of the controls. The data indicate that the effects of prenatal protein malnutrition on the mandible and long bone were quite different. Differential effects of the hormone, for example, on other organ systems have been documented (17).

Although calcium contents in the mandible and long bone of the recovered group at day 15 were about the same as that of the controls, calcium uptake of the bones of this group was much greater than the controls. Calcium uptake was even greater at day 15 in both bones by feeding continuously a 10% protein diet. In contrast, calcium contents in the mandible and long bone of this group were 19% and 53% lower, respectively, compared to that of the control group. It could be conceivable that this is due to the accelerated breakdown of the hard tissues. Since calcium uptake of the mandible and long bone of the postnatally malnourished pups has not shown any differences compared to that of the controls at day 15 (13), nutritional stress when imposed during both the pre- and postnatal period as in the present study might have somewhat different effects on the bone metabolism as compared to the postnatal stress alone (13). This seems also to apply to collagen synthesis.

When a 10% protein diet was fed continuously, both collagen contents of the mandible and long bone showed 28% and 35% less compared to that of the controls. However, collagen synthesis in the mandible and long bone of this group was greater also at day 15. The postnatal protein-energy malnutrition alone has again shown decreased collagen synthesis at day 15 (13).

Decreased collagen contents of the mandible in the prenatally malnourished offspring at day 1 showed the complete recovery of collagen contents and the rate of collagen synthesis was the same as that of the controls by day 15, suggesting that prenatal protein malnutrition on bones could be recovered well when proper refeeding is initiated early enough.

The present studies have demonstrated that prenatal malnutrition which affects the body weights as well as hard tissues can be recovered by day 15. It has been reported that limb bone contrary to the skull bone may not recover completely from protein-energy malnutrition by a proper refeeding (15). A different experimental design compared to the present study would be the cause of this discrepancy. However, if severe malnutrition is imposed on the prenatal period, the above conclusion may not necessarily hold true, although it is difficult to test such an hypothesis because of the extremely high postnatal mortality rate of the offspring.

ACKNOWLEDGEMENTS

This study was supported in part by the Research and Grants Committee of Louisiana State University Medical Center.

REFERENCES

1. Younoszai, M.K., Swanson, J.D. and Ranshaw, J. Intestinal disaccharidases in newborn and mother rats: Effect of dietary protein deficiency during pregnancy. Am. J. Clin. Nutr. 31: 931-937, 1978.

2. Endo, S., Kamori, K. and Niyama, Y. Effect of protein depletion during pregnancy and protein repletion after parturition on cellular growth in rats. J. Nutr. Sci. Vitaminol. 20: 143-152, 1974.

3. Zeman, F.J. Effect of protein deficiency during gestation on postnatal cellular development in the young rat. J. Nutr. 100: 530-538, 1970.

4. Platt, B.S. and Stewart, R.J.C. Transverse trabeculae and osteoporosis in bones in experimental protein-calorie deficiency. Br. J. Nutr. 16: 483-496, 1962.

5. Ramalingaswami, V. and Deo, M.G. Experimental protein calorie malnutrition in the rhesus monkey. In: Calorie Deficiencies and Protein Deficiencies, R.A. McCance and E.M. Widdowson (eds.) J.A. Churchill, London 1968, pp. 265-275.

6. Winkler, M.M., Porter, J.R. and Nakamoto, T. Prenatal protein-energy malnutrition in the growth of mandibles and long bones in neonates. Submitted for publication.

7. Angulo-Colmenares, A.G., Vaughan, D.W. and Hinds, J.W. Rehabilitation following early malnutrition in the rat: Body weight, brain size, and cerebral cortex development. Brain Res. 169: 121-138, 1979.

8. Williams, J.P.G. and Hughes, P.C.R. Muscle growth during neonatal undernutrition and subsequent rehabilitation in the rat. Acta Anat. 101: 249-254, 1978.

9. Tulp, O.L., Tyzbir, R.S., Morse, S.G. and Horton, E.S. Effect of prenatal protein deprivation and subsequent rehabilitation on adipose tissue development in rats. Nutr. Rep. Int. 22: 135-146, 1980.

10. Ten state nutrition survey in the United States - 1968-1970. U.S. Department of Health, Education and Welfare. Publication No. (HSM) 72-8130-8134, 1972.

11. Osofsky, H.J. Relationships between nutrition during pregnancy and subsequent infant and child development. Obst. and Gyn. Surv. 30: 227-241, 1975.

12. Nakamoto, T. and Miller, S.A. Effect of protein-energy malnutrition on the growth of mandible and long bone in newborn male and female rats. J. Nutr. 107: 983-989, 1977.

13. Nakamoto, T. and Miller, S.A. The effect of protein-energy malnutrition on the development of bones in newborn rats. J. Nutr. 109: 1469-1476, 1979.

14. Dixson, W.J. and Massey, F.J. Introduction to Statistical Analysis. McGraw-Hill, New York, 1969. pp. 109-126.

15. Lee, M. Skeletal growth and body size of rats during protein-energy malnutrition and during rehabilitation. Nutr. Rep. Int. 13: 527-533, 1976.

16. Nakamoto, T. and Miller, S.A. Physical and biochemical changes of the mandible and long bone in protein-energy malnourished newborn rats. J. Nutr. 109: 1477-1482, 1979.

17. Clark, A.F. and Vigals, P.J. Experimental corticosterone myopathy. Effect on myofibriller ATPase activity and protein degradation. Muscle and Nerve 2: 265-273, 1979.

NUTRITION RESEARCH, Vol. 1, pg. 393-397, 1981
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SERUM ENZYME ACTIVITIES IN MICE FED A HIGH LEVEL OF ASCORBIC ACID[1]

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ABSTRACT

Serum enzyme activities were compared in Swiss albino male mice receiving either untreated drinking water or drinking water containing 250 mg L-ascorbic acid per 100 ml. Mice on the ascorbate regimen consumed approximately 12 mg ascorbic acid per day. After four weeks, mice receiving the supplementary ascorbic acid had higher mean serum lactate dehydrogenase activity than those receiving untreated water. Supplementary ascorbic acid appeared not to influence serum amylase, lipase, glutamic oxaloacetic transaminase, glutamic pyruvic transaminase, acid phosphatase, or alkaline phosphatase activities.

KEY WORDS: ascorbic acid, serum enzymes, enzyme analysis

INTRODUCTION

Large doses of ascorbic acid have been reported to have several effects in laboratory animals. Nandi and coworkers (1) found that rats receiving large doses of ascorbic acid excreted less urinary histamine when exposed to stressful conditions than nonsupplemented animals. Large doses of ascorbic acid reduced the growth retardation caused by organophosphorus insecticides in rats (2). Data from other studies indicate that large doses of ascorbic acid may enhance resistance to toxic effects of heavy metals (3,4).

Several blood constituents also appear to be influenced by large doses of ascorbic acid. High serum levels of ascorbic acid have been noted in rats consuming large amounts of ascorbic acid (5). Marcusen and Heninger (6) observed lower thyroxine and thyroid-stimulating hormone in sera of rats receiving large amounts of ascorbic acid than in control rats. BALB/cJ mice fed supplementary ascorbic acid exhibited higher circulating interferon after stimulation with murine leukemia virus than nonsupplemented mice (7). Large doses of ascorbic acid increased plasma cholesterol in guinea pigs (8).

In our study, we used Swiss albino mice to investigate effects of high ascorbic acid intake on serum enzyme activities.

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[1]Supported in part by NIH Biomedical Research Grant RR07036, and the Kansas Agricultural Experiment Station, Contribution No. 81-485-j.

MATERIALS AND METHODS

Healthy weanling male mice weighing 11 ±2g were obtained from our departmental breeding colony of random-bred Swiss albino mice. They were housed in polypropylene cages in a temperature-controlled room (22° C) with a 12-hour light-dark cycle. Mice had free access to a stock diet (Purina Laboratory Chow, Ralston Purina Co., St. Louis, Mo.) and deionized water throughout the experimental period unless otherwise stated.

Mice on the ascorbic acid regimen received fresh daily 250 mg L-ascorbic acid per 100 ml drinking water. That diet had been used previously for mice (7,9,10) and we found that it did not restrict water intake. Animals in the control group received drinking water without ascorbic acid. Mice consumed approximately 5 ml water per day equivalent to 12 mg ascorbic acid each by the supplemented mice. After 4 weeks on that regimen, they were fasted overnight and sacrificed by cardiac puncture under light ether anesthesia. Blood was allowed to clot at room temperature, and obtained sera was refrigerated at 4° C. Care was taken to prevent hemolysis during sampling procedures. All control and experimental animals used for a specific enzyme determination were sacrificed the same day.

The serum was then used to measure the activities of lactate dehydrogenase (EC 1.1.1.27) (11), amylase (EC 3.2.1.1) (12), lipase (EC 3.1.1.3) (13), glutamic oxaloacetic transaminase (EC 2.6.1.1) (14), glutamic pyruvic transaminase (EC 2.6.1.2) (14), acid phosphatase (EC 3.1.3.2) (15), and alkaline phosphatase (EC 3.1.3.1) (15). All enzyme assays were performed on the day of sacrifice except those for the transaminases and lipase which were performed the following day. Blood content of ascorbic acid was assayed by the 2,4 dinitrophenylhydrazine method of Bessey (16) on the day of sacrifice.

Because ascorbic acid interferes with some diagnostic assays (17), we also tested the effect of adding 3 mg/100 ml ascorbic acid to pooled sera for each of our analytical procedures. We selected that amount because mice receiving the ascorbic acid regimen had blood levels similar to that concentration.

All differences between control and ascorbic acid-supplemented groups were analyzed by Students *t* test (18).

RESULTS AND DISCUSSION

Results are summarized in Table I. The ascorbic acid-supplemented mice had lower fasted body weights ($P<0.05$) and higher blood ascorbic acid levels ($P<0.01$) than control animals. We previously observed a growth restriction in Swiss albino mice receiving this ascorbate regimen (19). Marcusen and Heninger (6) found that rats receiving large doses of ascorbic acid had lower body weights than control animals.

In our study, ascorbic acid-treated mice had higher mean serum lactate dehydrogenase activity than control mice. Dietary ascorbic acid did not appear to influence the activities of the other enzymes studied.

Data from previous reports indicate that large doses of ascorbic acid can interfere with autoanalyzer estimations of serum transaminase and lactate dehydrogenase activities (20). In our study, ascorbic acid added to pooled sera during analytical procedures did not influence the activities of any

TABLE I

Animal Responses to Control and Ascorbic Acid-supplemented Diets

Measurement	Control	Control + ascorbic acid	p
	Mean ± S.E.M.		
Fasted weight *(g)*	28.9±.6 (67)[1]	27.2±.5 (70)	0.05
Blood ascorbic acid *(mg/100 ml)*	2.48±.14 (6)	3.27±.09 (6)	0.01
Serum enzyme activities:			
Lactate dehydrogenase *(Berger-Broida units/ml)*	448±42 (8)	588±46 (9)	0.05
Amylase *(Somogyi units/ml)*	29.0±1.8 (8)	29.3±1.3 (10)	N.S.
Lipase *(Sigma-Tietz units/ml)*	1.4±.07 (12)	1.4±.07 (11)	N.S.
Glutamic pyruvic transaminase *(Sigma-Frankel units/ml)*	29.8±2.0 (10)	31.8±1.3 (9)	N.S.
Glutamic oxaloacetic transaminase *(Sigma-Frankel units/ml)*	92.3±4.8 (9)	86.9±3.4 (12)	N.S.
Alkaline phosphatase *(Bessey-Lowry units/l)*	3.7±0.3 (10)	4.5±0.4 (9)	N.S.
Acid phosphatase *(Bessey-Lowry units/l)*	0.24±.02 (10)	0.28±.03 (10)	N.S.

[1]Number of animals in each group indicated in parenthesis

enzyme studied. The discrepancies may arise from differences in analytical methods, level of blood ascorbic acid, and species.

Measurements of serum enzyme activities are used clinically to diagnose various diseases in humans. For example, elevated amylase and lipase activities frequently are seen in subjects with pancreatic disease; and elevated lactate dehydrogenase, transaminase, and phosphatase activities may be observed in subjects with certain liver diseases (21). Some evidence suggests that high doses of ascorbic acid may damage the pancreas (22). We recently found that mice receiving large doses of ascorbic acid had smaller livers than control animals (19), so we suspected that serum enzyme activities would be altered in the animals we used in this experiment. However our data showed no enzyme-activity pattern indicating either pancreatic or liver malfunction in ascorbic acid-treated mice.

Ascorbic acid-fed mice had higher mean lactate dehydrogenase activity than control animals. A possible explanation is that large doses of ascorbic acid affected the release of this enzyme from tissues without causing any detectable tissue damage. Mengel and Greene (23) found that the erythrocytes of humans receiving large doses of ascorbic acid had increased sensitivity to the lytic effects of hydrogen peroxide. Also, ascorbic acid is a strong

reducing agent that activates several oxidation-reduction reactions (24). Perhaps the increased lactate dehydrogenase activity in ascorbic acid-treated mice was a long-term response to high serum ascorbic acid. Further research is needed to clarify this observation.

There is little information on the effects of large doses of ascorbic acid on serum enzyme activities. Our study indicated that serum lactate dehydrogenase activity was influenced by high ascorbic acid intake in mice.

ACKNOWLEDGMENT

The assistance of Dr. Arthur D. Dayton, Department of Statistics, is gratefully acknowledged.

REFERENCES

1. NANDI, B.K., SUBRAMANIAN, N., MAJUMDER, A.K. and CHATTERJEE, I.B. Effect of ascorbic acid on detoxification of histamine under stress conditions. Biochem. Pharmacol., 23:643-647, 1974.

2. CHAKRABORTY, D., BHATTACHARYYA, A., MAJUMDAR, K., CHATTERJEE, K., CHATTERJEE, S., SEN, A. and CHATTERJEE, G.C. Studies on L-ascorbic acid metabolism in rats under chronic toxicity due to organophosphorus insecticides: Effects of supplementation of L-ascorbic acid in high doses. J. Nutr. 108:973-980, 1978.

3. HILL, C.H. Studies on the ameliorating effect of ascorbic acid on mineral toxicities in the chick. J. Nutr., 109:84-90, 1979.

4. FOX, M.R.S. Protective effects of ascorbic acid against toxicity of heavy metals. Ann. N.Y. Acad. Sci., 258:144-149, 1975.

5. BEHRENS, W.A. and MADERE, R. Effects of high ascorbic acid intake on the metabolism of catecholamines in the rat. J. Nutr., 110:720-724, 1980.

6. MARCUSEN, D.C. and HENINGER, R.W. Effect of ascorbic acid on the pituitary-thyroid system in the rat. J. Endocrinol., 70:313-314, 1976.

7. SIEGEL, B.V. Enhanced interferon response to murine leukemia virus by ascorbic acid. Infec. Immun., 10:409-410, 1974.

8. HOLLOWAY, D.E. and RIVERS, J.M. Influence of chronic ascorbic acid deficiency and excessive ascorbic acid intake on bile acid metabolism and bile composition in the guinea pig. J. Nutr., 111:412-424, 1981.

9. FRITH, C.H., RULE, J. and KODELL, R.L. The effects of ascorbic acid on the induction of urothelial lesions in mice by 2-acetylaminofluorene. Tox. Lett., 6:309-318, 1980.

10. SCHLEGEL, J.U., PIPKIN, G.E., NISHIMURA, R. and SCHULTZ, G.N. The role of ascorbic acid in the prevention of bladder tumor formation. Trans. Am. Assoc. Genitourin. 85-89, 1969.

11. CABAUD, P.G. and WROBLEWSKI, F. Colorimetric measurement of lactic dehydrogenase activity of body fluids. Amer. J. Clin. Path., 30:234-236, 1958.

12. SOMOGYI, M. Modification of two methods for the assay of amylase. Clin. Chem., 6:23-25, 1960.

13. TIETZ, N.W. and FIERECK, E.A. A specific method for serum lipase determination. Clin. Chim. Acta., 13:352-358, 1966.

14. REITMAN, S. and FRANKEL, S. A colorimetric method for the determination of serum glutamic oxalacetic and glutamic pyruvic transaminases. Amer. J. Clin. Path., 28:56-63, 1957.

15. BESSEY, O.A., LOWRY, O.H. and BROCK, M.J. A method for the rapid determination of alkaline phosphatase with five cubic millimeters of serum. J. Biol. Chem., 164:321-329, 1946.

16. BESSEY, O.A., LOWRY, O.H. and BROCK, M.J. The quantitative determination of ascorbic acid in small amounts of white blood cells and platelets. J. Biol. Chem., 168:197-205, 1947.

17. HERBERT, V. The rationale of massive-dose vitamin therapy. In: Proceed. West. Hemisphere Nutr. Congress IV, pp. 84-91, Publish. Sciences Group, Acton, MA, 1975.

18. SNEDECOR, G.W. and COCHRAN, W.G. Statistical Methods. Iowa State University Press, Ames, IA, 1976.

19. GRUNEWALD, K.K. and MITCHELL, L.K. In review, 1981.

20. BARNESS, L.A. Safety considerations with high ascorbic acid dosage. Ann. N.Y. Acad. Sci., 258:523-527, 1975.

21. DAVIDSOHN, I. and HENRY, J.B. (eds.) Todd-Sanford Clinical Diagnosis by Laboratory Methods, 15th ed. W.B. Saunders Co., Philadelphia, PA.

22. PATTERSON, J.W. The diabetic effect of dehydroascorbic and dehydroisoascorbic acids. J. Biol. Chem., 183:81-88, 1950

23. MENGEL, C.E. and GREENE, H.L., Jr. Ascorbic acid effects on erythrocytes. Ann. Intern. Med. 84:490, 1976.

24. KUTSKY, R.J. Handbook of Vitamins and Hormones. Van Nostrand Reinhold Co., New York, N.Y., 1973.

NUTRITION RESEARCH, Vol. 1, pg. 399-418, 1981
0271-5317/81/040399-20$02.00/0 Printed in the USA.
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MATURATION OF INTESTINAL HOST DEFENSE: AN UPDATE

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ABSTRACT

Factors present in the intestinal lumen and mucosa act as a first line of defense against ingested pathogens and noxious substances; the liver functions as a second line of defense. Intestinal and hepatic host defense mechanisms are immature early in life. During this critical period of compromised host defense, colostrum and breast milk passively protect the infant from infectious agents and antigens. In addition, colostrum and breast milk may stimulate the development of intestinal host defense mechanisms.

KEY WORDS: Host defense, maturation, intestine

The intestine is a complex organ with diversified functions which include digestion, absorption and excretion. This organ also functions to protect the host from a large number of chemicals, potential antigens and pathogens. In this review I will discuss intestinal host defense mechanisms and the developmental aspects of these mechanisms. I have relegated intestinal host defense arbitrarily into four categories and will discuss each separately (Table 1).

Table 1

Host Defense Mechanisms

Luminal Defense

- Gastric pH and Proteolysis
- Intestinal Flora
- Bile Acids

Mucosal Defense

- Gastrointestinal Associated Lymphoid Tissues
- Mucin-Producing Cells
- Macrophages
- Enterocytes
- Mast Cells
- Contractile Cells

Hepatic Defense

Passive Defense

LUMINAL DEFENSE

Gastric pH and Proteolysis: Ingested substances are rapidly mixed with

water, electrolytes, acid and pepsin in the stomach. The pepsin in the presence of gastric acid initiates protein digestion, however, it is in the small intestine that the bulk of protein digestion occurs. Proteins are broken down in the intestinal lumen by pancreatic enzymes and enzymes contained in the brush border of the intestinal epithelium. Trypsinogen, one of the more important pancreatic enzymes, in the presence of the brush boder enzyme enterokinase is converted to its active form, trypsin. Trypsin then accelerates the conversion of trypsinogen to trypsin and also catalyzes reactions yielding chymotrypsin, carboxypeptidase A and carboxypeptidase B (1,2). These activated proteolytic enzymes are important in adequately hydrolyzing exogenous proteins which may be potential antigens.

Both acid secretion by the stomach and gastrointestinal proteolysis appear to be important host defense mechanisms. The importance of gastric acid to host defense is underscored by studies which show that gastric acid must be at least partially neutralized with bicarbonate in order for human volunteers to develop diarrhea after ingesting Vibrio cholerae. Music et al (3) used healthy informed volunteers and challenged them orally with toxin-producing Vibrio cholerae. Diarrhea was induced by 10^4 organisms with bicarbonate and by 10^8 organisms without it. The duration of the buffer effect from the bicarbonate could be correlated with susceptibility to disease induction. Further evidence of the protective effect of gastric acid has been provided by Kraft (4). He reported increased circulating anti-bovine serum albumin (BSA) antibodies in adults with achlorhydria and suggested that these individuals do not adequately hydrolyze the common food antigen BSA in the stomach, and it therefore may be present in greater concentrations for absorption by the small intestine. The immune system is then triggered and antibodies produced by the increased quantities of circulating BSA.

Proteolytic activity in the small intestine also appears to be important in intestinal host defense. Walker (5) has shown that the breakdown of ^{125}I-bovine serum albumin by jejunal gut sacs from pancreatic duct-ligated rats is significantly decreased in comparison to sham operated controls. In addition, treatment of pancreatic duct-ligated rats with pancreatic extracts prior to removal of the gut sacs, leads to an increase in digestion of ^{125}I-BSA by in vitro gut sacs. These findings suggest that pancreatic enzymes adsorbed to the surface of the intestine contribute to proteolysis of antigens. More recently, Saffran and colleagues (6) administered lysine vasopressin (LVP) by gavage to rats simultaneously with the trypsin inhibitor Trasylol® and noted an increased anti-diuretic effect in these animals compared to control animals given LVP alone. This finding extends Walker's earlier in vitro studies and demonstrates that in vivo inhibition of pancreatic enzymes leads to increased amounts of vasopression being transported across the intestine and into the blood stream. We have substantiated Saffran's findings in newborn rabbits gavaged with Trasylol® and BSA or saline and BSA. The Trasylol®-BSA treated animals have two times the amount of immunoreactive BSA per ml of plasma four hours after the gavage dose of BSA as the animals given saline-BSA (7).

Development: Acid secretion by the stomach is decreased in the newborn period. Gastric H+ output in meq/hr/kg the first month of life in human infants is approximately one-tenth that of adults (8). Furthermore, proteolysis of ingested proteins may also be decreased early in life. Antonowicz and Lebenthal (9) noted that enterokinase activity in full-term human infants is only 17% that found in children between one and four years of age. This is in agreement with the observation that newborn infants have approximately one-tenth the intestinal tryptic activity found in children nine months of age and older (10). These studies which suggest that proteolytic enzyme activity in the intestine of newborns is decreased are supported by recent clinical observations concerning the etiology of necrotizing enteritis in New Guinea. This disease which occurs in infants and children is caused by a beta toxin elaborated by Clostridia welchii, type C (11). Undigested toxin produced by the organism interacts with the intestinal epithelium to cause severe inflammation. Infants and chil-

dren appear to be predisposed to the disease because of an inability of their intestinal enzymes to adequately digest the toxin.

Intestinal Flora: Several reviews have suggested that normal bacterial and viral populations of the gastrointestinal tract may be a deterrent to the overgrowth of pathogens which may be of potential importance in infectious disease states (12,13,14). The importance of a well-balanced intestinal microbial population has been clearly demonstrated in experiments involving germ-free animals. In the germ-free state, animals suddenly exposed to the normal external environment may rapidly develop fulminant gastroenteritis or systemic infection. Studies have also shown that the quantity and quality of indigenous intestinal flora are of equal importance as a defense against pathogens (15). The interactions between the normal flora and pathogens may take the form of competition for substrates, alteration in the microenvironment for optimum growth (lowered pH, decrease in oxidation-reduction potential, etc.) or the production of antibacterial substances (16,17). Whatever the specific mechanisms of defense, it is apparent that indigenous microorganisms are important in the control of pathogen overgrowth within the intestinal tract.

Development: After birth, the newborn infant's intestine becomes rapidly colonized with a mixture of organisms which compromise the normal or indigenous microbial population of the gut. The nature of the intestinal flora of the newborn human is determined by a number of factors, including diet (nursing versus bottle feeding) and competitive interactions of organisms (18).

Bile Acids: Many microorganisms grow poorly in media containing bile salts (19). This observation suggests that the paucity of bacterial growth within the small intestine may be due in part to the high concentration of bile at that site. Although actual inhibition of anaerobic bacterial proliferation by bile acids in vivo has been demonstrated, the inhibition of proliferation does not correlate directly with concentrations of bile salts and intestinal organisms survive in succus entericus containing bile (20).

Development: The bile acid pool differs quantitatively in the newborn human compared to the adult. The pool and luminal concentration of bile acids in the intestine is decreased in the infant (21). The lower concentration of bile acids in the small intestine is thought to be due to decreased secretion of bile acids by the liver and decreased ileal mechanism for active bile acid absorption, not allowing for effective conservation of bile acids (22). Bile acids differ qualitatively in the newborn human in that the percentage of bile acids conjugated with taurine (60%) exceeds the glycine conjugates (40%). The reverse is true for the adult when 76% of bile acids are glycine conjugates and 24% taurine (21). Whether these quantitative and qualitative changes in the bile acid pool during infancy affect bacterial growth or intestinal host defense early in life is not known.

MUCOSAL DEFENSE

Gastrointestinal Associated Lymphoid Tissues (GALT): Lymphocytes are scattered throughout the mucosa of the gastrointestinal tract and between the epithelial cells covering the intestinal villi. In addition, organized aggregates of lymphocytes, collectively termed the gastrointestinal-associated lymphoid tissues, are also present. The most frequently cited examples of GALT are the tonsils, Peyer's patches, and the appendix. It is generally considered that the aggregated lymphoid structures are the principle sites in the mucosa where interaction of antigen from the gut lumen and circulating lymphocytes takes place, whereas the cells in the lamina propria and epithelium are the effector cells which carry out immune responses (23).

Gastrointestinal-associated lymphoid tissues have many features in common with either secondary or peripheral lymphoid tissues such as the spleen and lymph nodes. They may be subdivided roughtly into T and B areas in which either T or B lymphocytes predominate (24). When selectively enriched populations of labeled T or B lymphocytes are injected intravenously they can be

shown to migrate to specific T or B areas. Lymphocytes appear to migrate through the GALT by similar routes and processes as occurs in other peripheral lymphatic tissue (25).

Peyer's patches, located in the ileum of man, consist of clusters of lymphoid follicles having well-defined structures. Recent studies have demonstrated that a specialized epithelial cell overlying Peyer's patches may facilitate the access of antigens to intestinal lymphoid tissues (26,27,28). Morphologically these microfold-cells (M-cells) as they are called, have a paucityof microvilli, a poorly developed glycocalx and an absence of lysosomal organelles. Horseradish peroxidase has been used by Owen (29,30) to study antigen uptake by M-cells (Figure 1). The marker is taken up into the specialized cells and rapidly released into the interstitial space and processed by lymphoid cells circulating through Peyer's patches. This mechanism of antigen transport in the gut appears to represent an important specialized access route for ingested antigen to reach lymphoid tissues and thereby stimulate the local and distant immune system. Not only are intact macromolecules processed by M - cells, but also viral particles. Wolfe (31) innoculated reovirus into the intestinal lumen of mice, and using electron microscopy noted that viruses were adhering to the surface of the intestinal M-cells but not to other epithelial cells 30 minutes later. At one hour virus particles were seen in the M-cell cytoplasm and were associated with mononuclear cells in the intercellular space adjacent to the M-cell. The studies of Owens, Wolf and others (25,26,27,28) indicate that these specialized M-cells may be an important mechanism by which macromolecules and pathogens not digested or destroyed in the proximal small intestine gain access to lymphoid tissue of the distal gut.

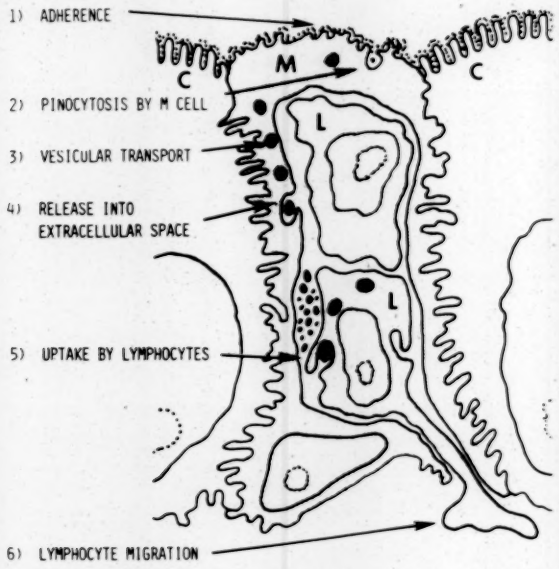

FIG. 1

Schematic representation of the different stages involved in the transport of horseradish peroxidase (HRP) by M-cells from the intestinal lumen to the intraepithelial lymphocytes (L). The columnar cells (c) adjacent to the M-cells are identified. (Reproduced by permission, Reference 29)

The most studied mucosal defense mechanism of GALT is the synthesis and release of a special form of the immunoglobulin A (IgA), secretory IgA (SIgA), which is the predominant immunoglobulin of gastrointestinal secretions (32,33). Immunoglobulin A is produced by plasma cells in the lamina propria and passed into the gut lumen after linkage to secretory piece in the epithelial cells (12,32). Plasma cells which produce IgA reach the mucosa after a complicated migration of precursors initially stimulated in the distal ileum via the specialized M-cells. The antigens are in some way processed and lymphocytes sensitized and released, leading to the presence in the thoracic duct lymph of lymphocytes which stain for specific IgA antibody. Pierce (28) has studied this migration using cholera toxin and toxoid in rats in which antitoxin-containing cells were demonstrated in thoracic duct lymph following the intraduodenal presentation of toxin. He further reported that these lymphoid cells could eventually be found in the lamina propria, particularly at sites in which the antigen was first presented. This study and others demonstrate that cells stimulated by antigen to differentiate into IgA producers have been attracted back or have "homed back" to the site where the antigen was originally present. This traffic pattern of migrating cells from the gut back to intestinal mucosal surface also occurs between the gut and the mucosa of the bronchus and mammary gland in the lactating animal (34).

The major host defense mechanism of antibody of both SIgA (35) and fragments of other Ig classes (36) in the intestinal lumen is due to binding of antibody to antigen (immune exclusion), thereby preventing attachment to the epithelial surface. It has been suggested that IgA may also coat the intestinal epithelium and prevent bacterial adhesion to the epithelial surface and suppress the growth of certain bacterial pathogens (37,38,39). The search for other potential activities by this immunoglobulin, such as opsonization and complement fixation, has been largely disappointing (23).

The importance of IgA in host defense at mucosal surfaces has been questtioned because of the well being of most patients with selective IgA deficiency in their serum and secretions. However, in this group of patients, it appears that increased numbers of IgM plasma cells in the mucosa compensate for IgA deficiency by secreting IgM into the intestinal lumen (40). The IgM appears to bind to secretory component in a manner similar to IgA as it passes through the epithelial cells into the lumen (41).

Development: Serum immunoglobulin concentrations at birth in humans are significantly different from adult concentrations. Serum IgG concentration is higher and IgA lower early in life (42). In addtition there is a reduction in salivary (43) and stool (44) IgA in newborns compared to older children and adults.

Burgio et al (45) measured not only secretory IgA in the saliva of healthy human subjects from two months to 27 years of age, but also free secretory component and antibody titers against E.coli O antigens in the saliva. He found a physiologic deficiency of SIgA and secretory antibodies in infancy and childhood and speculated that this deficiency may well play a role in the pathogenesis of infectious diseases of the respiratory and gastrointestinal tract frequent in this age group. His study is supported by the findings of Perkkio and Savilahti (46). These investigators noted a deficiency of immunoglobulin-containing cells in the gut of newborn humans up to 12 days of age. The reduction in immunoglobulin producing cells and SIgA in the intestine of infants may account for the increased incidence of infection at mucosal surfaces early in life and may also be a factor in the development of atopic disease (47,48,49).

Levels of serum, salivary and stool IgA are known to be decreased in the newborn human. In addition, the ability of newborns and particularly premature newborns to mount an immune response may be compromised. To evaluate immune response in human infants Reiger and Rothberg (50) fed bovine serum albumin to newborns delivered at different gestational ages and followed the development of serum anti-BSA-antibodies. Four of five infants 35 weeks gestation or older developed antibody to BSA whereas none of eight infants younger than

35 weeks produced anti-BSA. However, factors other than gestational age appear to be important for a normal immune response. Studies have clearly demonstrated that newborn and germ-free animals exposed to a paucity of intestinal antigens have a striking absence of antibody producing cells (51,52,53,54). These observations emphasize the important role of age and local antigenic exposure of GALT in stimulating differentiation of lymphoid tissue which is capable of synthesizing antibodies at intestinal mucosal surfaces. Additional factors also appear to be necessary to complete the process of immune maturation. These factors include T lymphocytes, macrophages (32) and possibly soluble adjuvants in breast milk (55,56).

Maternal antibodies, especially IgG, protect the newborn animal during the susceptible neonatal period. These antibodies are transported from mother to developing animal in utero in humans and rabbits (32). In other species maternal IgG is acquired not in utero, but post-natally from breast milk. In newborn sheep, cows and horses IgG is transported intact from the intestinal lumen into the blood (32). Although the absorption and transport of maternal IgA from breast milk is not thought to occur in man, serval studies oppose this view. Iyengar and Selvaraj (57) examined serum immunoglobulins from human infants at birth to five days of age and found that levels of all immunoglobulins (IgG, IgM, IgA) on the fifth day were significantly higher in the serum of colostrum-fed infants compared to those who did not receive colostrum. These investigators suggest that immunoglobulins present in colostrum are absorbed from the intestinal tract of the newborn human. Alternatively, factors present in breast milk may stimulate the production of endogenous antibodies. In another study, Ogra et al (58) administered in a single feed to newborn humans, human colostrum rich in IgA antibody against poliovirus. Shortly thereafter anti-poliovirus antibody was detected in the blood of three of seven infants whose cord blood had been negative for antibody against polio. These observations suggest that orally ingested maternal immunoglobulins may be absorbed in human infants and transported across the intestine and into the blood in the immediate neonatal period.

The dilemma of whether intact immunoglobulins are absorbed and transported from breast milk by the human neonatal intestine has not been resolved completely. However, the protective value of breast milk antibodies has been documented by Hilpert (59). He administered anti-E.coli bovine colostrum to infants under seven months of age with E.coli enteritis and noted accelerated clinical and bacteriologic improvement in these infants compared to control infants receiving bovine colostrum with no anti-E.coli activity. Additional studies are necessary before this mode of treatment can be accepted without risk for human neonates.

Mucin-Producing Cells: Intestinal mucins, or glycoproteins, are complex macromolecules containing a protein core and side chains of several carbohydrates. Mucin production has been demonstrated to take place in both goblet cells and intestinal epithelial cells. Forstner (60) showed that a large part of intestinal mucin arises from epithelial cells. However, mucins from goblet cells, epithelial cells and other glycoproteins probably contribute to the mucin layer of the intestine (61).

Edwards (62) has suggested that intestinal mucins may be a medium in which most macromolecules are insoluble, whereas small molecules are soluble and free to diffuse. Therefore, he speculates that the prime role of mucins may be to exclude macromolecules and larger particles from contact with cell membranes. He points out that IgA occurs with mucins in many secretions (63). In fact, IgA molecules have a mucin-like glycoprotein sequence at the hinge region between their Fc and Fab sub-units (64,65). This observation raises the possibility that IgA sits at the interface between mucin and overlying fluid with its mucin-like portion in the mucin phase and the rest of the molecule in the overlying aqueous phase, like a detergent or a phospholipid at the interface between a lipid and a water phase. The IgA would then form a monolayer on the surface of the mucin, where it is needed in defense against potential antigens

and pathogens. Edward's suggestion that mucin retards macromolecular absorption is supported by some experimental observations. Nimmerfall and Rosenthaler (66) have noted that diffusion of a compound through the hydrated mucin network is likely to depend on the charge, hydration radius of the molecule and the ability of the molecule to form hydrogen bonds. In addition they have found that intestinal absorption of molecules in the rat correlates inversely with molecular weight. Hence, high molecular weight molecules are retarded in their passage through the mucin layer and therefore are not absorbed as extensively as smaller molecules. In addition to possibly retarding the penetration of macromolecules and potential antigens, mucins may also act as a physical barrier to enterotoxins. There are reports in the literature that the shigella (67), staphylococcal (68), klebsiella (69) and cholera (70) enterotoxins stimulate mucin release and/or synthesis. The increased amount of mucin in response to an enterotoxin may be a protective mechanism of the host. In experiments to determine if mucins protect the intestine against enterotoxins Strombeck and Harrold (71) found that gastric and intestinal mucins bind and precipitate choleragen. However, they showed that only gastric mucin inhibited the secretory stimulation of toxin in the intestine.

Development: Meconium, a mucin-like substance which coats the gastrointestinal tract at birth contains predominantly glycoprotein with little fat. It is generally passed within 24 hours of birth in more than 90% of newborns (72, 73). Several investigators have noted that intestinal macromolecular uptake (74) and transport (75) in rabbits are decreased the first 24 hours of life compared to 4-7 days later. One possible explanation is that this thick, viscous glycoprotein substance lining the intestine protects the infant animal the first few hours of life until colostrum is ingested.

Studies of small intestinal mucin in older animals suggest that the component glycoproteins vary along the crypts and villi of the small intestine (61,76). It is thus tempting to correlate the variations in the mucin along the crypts and villi with cell differentiation; neutral mucins are produced by immature cells in the crypts and as cells mature and migrate up the villi increased amounts of sialomucins are synthesized by the goblet and epithelial cells. This alteration in glycoprotein synthesis with cell maturation finds support in parallel changes observed in glycocyl and sialyl transferases in maturing intestinal epithelial cells (77). In addition to the variation in mucin composition along the crypt-villous axis, mucins may differ with age. It is interesting to note that in the human fetal small intestine, mucin around the seventh week is a predominatly PAS positive neutral mucin and not sialomucins, however, later in development sialomucins predominate (78,79).

Macrophages: Although macrophages of lung, peritoneal cavity, spleen and liver have been discussed at length (80), little published work has dealt directly with the origin, abundance and activity of intestinal macrophages. Intestinal macrophages reside in the small intestine associated with GALT, surrounding the crypts of Lieberkuhn and in the villous core. Although intestinal macrophages are similar to macrophages in other parts of the body they have not been shown to possess all of the characteristic nuclear and lysosomal morphology, phagocytic activity, lysosomal acid phosphatase, and membrane receptors for the third complement component and the Fc portion of the IgG molecule (25).

Macrophages carry out diverse functions, including phagocytosis, secretion, and partcipation in certain cell-mediated and humoral immune responses. Recent studies have shown that after transit through M-cells in the Peyer's patch epithelium, particulate matter reaches the subepithelial zone and is then engulfed by the macrophages of the region (81,82,83). By electron microscopy, subepithelial macrophages have the hallmarks of intense phagocytic activity. Sobhon (84) noted that the cytoplasm of subepithelial macrophages of mouse Peyer's patch tissue was rich in lysosomal bodies and that these macrophages appeared to be highly active, even in germ-free mice.

Macrophage secretory function may be just as important to host defense as phagocytosis. One group of macrophage secretory products, the lysosomal

enzymes, may be assumed to have important actions on the cells of intestinal tissues by analogy with their actions elsewhere. Lysosomal enzyme release by macrophage cells depends both qualitatively and quantitatively on the state of activation of macrophages and nearby lymphoid cells as well as on the presence of immune complexes (25). Phagocytosis, particularly of toxic indigestible material, may lead to the release of lysosomal enzymes capable of causing injury to surrounding tissues. Many types of extraintestinal inflammation have been postulated to originate in this way (85,86) and a role for macrophage lysosomal enzymes has been considered in a few varieties of intestinal inflammation. Abraham and collaborators (87) postulated that injury by macrophage lysosomal enzymes contributed to cecal ulceration in guinea pigs given iota-carrageenan in drinking water. They demonstrated abundant, acid phosphatase rich macrophages loaded with fibrillar carrageenan-like material in the lamina propria of iota-carrageenan treated animals. Among numberous causes suggested for Crohn's disease in humans is the release of cytotoxic enzymes (88). Wall et al (89), in considering the pathology of gluten-induced coeliac disease, suggested that lysosomal enzymes might be involved, but they incriminated lysosomes produced by intestinal epithelial cells rather than macrophages.

Many macrophages continuously produce lysozyme, an anti-bacterial enzyme that hydrolyzes complex aminopolysaccharides. In some intestinal disease states in humans, the lysozyme content of stools and intestinal mucin increases, presumably because of increased leukocyte and macrophage activity in the intestine (90,91). The increased amount of intestinal lysozyme may be important in retarding growth of bacterial pathogens.

In addition to producing lysosomal enzymes and lysozyme, macrophages have been shown to synthesize prostaglandins (92). Prostaglandins may influence intestinal motility, secretion, blood flow and mucin production (93) and have been isolated from all gastrointestinal tissues studied. Although both prostaglandin E (PGE) and prostaglandin F (PGF) are present in the GI tissues of man, PGE predominates; in the human stomach there is an approximately ten-fold greater synthesis of PGE than PGF (94). Cholera toxin, theophylline and cyclic AMP have all been shown to increase prostaglandin production in the GI tract and some investigators have suggested that cyclic nucleotides may mediate the GI effects of prostaglandins (93).

The notion that prostaglandins have a gastrointestinal "cytoprotective effect" was introduced by Robert (95). He showed that the administration of prostaglandin synthesis inhibitors to rats caused two types of lesions: acute gastric ulcers and chronic, multiple ileal and jejunal ulcers. He prevented the gastric lesions with oral or subcutaneous prostaglandins, including those without known antisecretory effects. These findings support a mechanism other than a reduction in acid environment in the protective effects of prostaglandins. Similarly, small intestinal ulcers were prevented, in a dose-dependent fashion, with either oral or subcutaneous prostaglandins.

There is also evidence that prostaglandins produced by macrophages have an important role in the regulation of lymphocyte function (92). Antigen-stimulated lymphocytes make lymphokines that activate macrophages and stimulate them to synthesize and release prostaglandins (96). Exogenous prostaglandins are potent inhibitors of several lymphocyte functions including mitogenesis, cytolysis, and antibody and lymphokine production. Since macrophages and monocytes appear to be the major source of prostaglandins produced in the immune response, it is reasonable to suppose that macrophage- and monocyte-derived prostaglandin may act as physiologic inhibitors of lymphocyte function (92).

Development: Additional studies are necessary to better understand the importance of phagocytosis by macrophages and the synthesis of lysosomal enzymes, lysozyme and prostaglandins to host defense in the developing animal.

Enterocytes: The composition of cell membranes may be important in host defense and has recently been studied in connection with bacterial adherence studies. The implicit premise of these adherence studies is that the adherence

of bacteria to epithelial membranes involves a specific "lock and key" interaction between molecular ligands on the surface of the bacteria with specific structures (receptors) on the surface of the tissues to be colonized.

Evidence that the adherence of bacteria to tissue surfaces is an important initial event in the infectious process is provided by two types of recent studies. In the first type of study, it has been shown that the ability of a particular strain of bacteria to adhere to epithelial cells *in vitro* parallels the ability to produce infections *in vivo*. Of particular note is the finding reported by Dr. T.K. Satterwhite and co-workers (97,98) that a colonization factor negative, nonadherent mutant of an enterotoxigenic strain of *E. coli* failed to produce diarrhea in human volunteers even though it retained its ability to produce enterotoxin. Moreover, the nonadherent mutant was cleared more rapidly from the gastrointestinal tract than the parent, adherent strain. These findings strongly suggest that in order to effectively deliver their diarrhea-producing toxins to the intestinal mucosal cells, the bacteria must be able to adhere. Attached organisms are able to deliver their toxin molecules to the host cell membranes at high concentrations because the molecules have only a short distance to travel and thereby avoid dilution and attack by destructive chemicals and enzymes present in the luminal fluids.

In the second type of study, the capacity of epithelial cells from infection-prone persons to bind bacteria *in vitro* has been examined. A number of investigators have shown, for example, that *E. coli* adhere in much greater numbers to the periurethral and uroepithelial cells of females who have had recurrent urinary tract infections than to the epithelial cells from females who have not had such infections (99). One of the most convincing arguments that bacterial adherence is required for bacterial infectivity comes from the study of diarrhea in piglets induced by K88 strains of *E. coli* (100). While the intestinal epithelial cells of susceptible piglets bind larger numbers of *E. coli*, the epithelial cells of resistant animals bind very few or none at all. Cross-breeding of resistant and susceptible pigs has shown that both the binding capacity of intestinal brush borders and the susceptibility to *E. coli* K88-induced diarrhea are autosomal dominant traits. Thus, the receptor for K88 strains of *E. coli* appears to be missing in the resistant pigs.

Development: Recent studies have indicated that cell membrane composition changes as the epithelial cell migrates up the villus (101,102,103), and as the animal ages (104,105,106). Changes in membrane composition may affect binding of bacteria and toxins to the cells. Drs. Jean Louis Bresson and Alan Walker (107) have studied isolated microvilli membranes from the intestine of newborn and adult rabbits. They have shown that the membrane protein/phospholipid ratio is dramatically decreased in newborn membranes compared to adults. In addition, there is increased cholera enterotoxin binding to newborn membranes compared to adult intestinal membranes. These studies of developmental differences in the intestinal surface may help us to better understand why newborn infants have such a high incidence of some types of infectious diarrheas.

Mast Cells: In rodents, evidence indicates that there are two distinct populations of mast cells in the gastrointestinal mucosa (108). The submucosa is replete with typical connective-tissue mast cells while there are atypical mast-like cells in the epithelium. Preliminary data suggests that humans may also have two distinct subpopulations of gastrointestinal mast cells. These two types of mast cells or "globule leukocytes" proliferate rapidly during certain parasitic infections (109), have IgE receptors on their surface and degranulate in response to antigen (109,110,111,112).

The number of recognized mast cell-derived mediators (i.e. histamine, superoxides, proteases, inflammatory factors) which may be involved in the pathogenesis of allergic disease has increased dramatically in the past decade (108). These molecules are clinically and biologically diverse, are discharged from the mast cell over an extended time period and generate both immediate and delayed allergic reactions.

Infestation of rats with Nippostronglyus brasiliensis increases IgE-laden mast cells up to ten times initial values (109,113). Mayrhofer (114) has noted that rats reinfected with the parasite have a reduced latent period of the response, heightened magnitude, and an increased local proliferation of mucosal mast cells. He suggested that this secondary response may be a manifestation of an adaptive immune response to the parasite. Additional studies are necessary to evaluate the importance of mast cells in host defense.

Development: Virtually nothing is known about the seeding and development of mast cells within the gastrointestinal tract.

Contractile Cells: The muscle cells of the muscularis mucosae and tunica muscularis externa are important for normal segmental and peristalic motion of the intestinal tract; and normal peristalsis is thought to be an important host defense mechanism (12). In fact, some investigators feel that peristalsis of the intestine is the most important factor in controlling proliferation of bacteria.

HEPATIC DEFENSE

The liver receives the major components of serum filtrate from the gastrointestinal tract, and approximately 15 per cent of the total liver mass is composed of reticuloendothelial cells (115). These reticuloendothelial cells, or Kupffer cells, have an enormous capacity to phagocytize noxious substances which are absorbed from the intestine and enter the portal circulation. However, the route of absorption may be critical. Haptens absorbed directly into the portal circulation will gain access initially to Kupffer cells, whereas an antigen such as bovine serum albumin absorbed via the lymphatics will reach other organs and stimulate an immune response (37). If absorbed through the portal vein, the normal liver seems to trap particulate antigen but not soluble antigen unless it is complexed with homologous antibody. Although the liver may be considered a "second line of defense" against invasion of biologically active substances (12), it may also play a central role in the development of tolerance (116).

In addition to the phagocytic function of the liver, recent studies in animals and man have demonstrated that the liver may clear the circulation of SIgA. These studies have demonstrated that SIgA is transported free (117) and complexed to various substances (118,119,120) from serum into bile ducts. In addition, ligation of bile ducts has been shown to increase the concentrations of SIgA in the serum. The hepatic transport of SIgA from serum to bile may not only be a means of eliminating antigen from the circulation, but it may also serve to bolster intestinal host defense by re-circulating IgA to the intestinal lumen.

PASSIVE DEFENSE

Studies of morbidity and mortality during infancy support the view that breast milk provides the newborn infant with passive protection from infection and allergy. Initial observations of the protective effect of breast milk were first made in the 1930s (121,122,123). In more recent years these observations were generally believed to be of little consequence in modern societies because of improved general sanitation (124,125). A renewed interest in breast-feeding has prompted a re-examination of the protective effect of human milk in reducing the incidence of infection (33,126,127,128). Cunningham (129) has demonstrated that breast-fed infants have fewer illnesses, and Winberg and Wessner (130) found that infants fed human milk have a lower incidence of septicemia. Host defense factors present in breast milk that may account for this lower incidence of morbidity and mortality include lymphocytes, macrophages, immunoglobulins, lysozyme, prostaglandin and lactoferrin (126-131). However, other factors in breast milk may be important to host defense.

Recently a substance isolated from breast milk cells has been reported to

act as a mitogen specifically for IgA producing cells (55,56). This mitogenic factor is present in milk collected from women who were less than five days post partum. In addition, the factor can be obtained from breast milk cells cultured for seven days. The factor stimulates IgA production in peripheral blood lymphocytes as effectively as pokeweed mitogen but differs distinctly from pokeweed mitogen in producing no changes in IgG and IgM production. It has been hypothesized that the breast milk substance may provide an active and passive role in providing immune protection to the newborn. The passive immune protection to the neonate may occur through stimulation of mammary gland lymphocytes to secrete IgA. Alternatively, the stimulatory factor may activate the neonate's own intestinal lymphocytes to produce IgA. Support for the latter hypothesis comes from the observation of increased IgA levels in the nasal secretions of breast-fed infants (132).

In addition to breast milk factors which may stimulate lymphocytes to produce IgA, other trophic factors in breast milk may accelerate intestinal development. Widdowson et al (133), reported that the small intestines of piglets given sow colostrum during the first twenty-four hours of life are substantially increased in weight and protein content compared to the small intestines of piglets fed only water. Heird (134), using isocalorically matched colostral- and formula-fed Beagle pups, found that small intestinal mucosal mass and DNA content increased significantly more in the group ingesting maternal colostrum. He suggested that colostrum contains an "epithelial growth factor" which stimulates epithelial cell turnover and maturation. Furthermore, Klagsbrun (135,136) has recently shown that human milk contains a mitogenic factor that stimulates DNA synthesis and cell division in mouse and human fibroblasts in vitro. He found that the mitogenic effect of human milk is 50-100 times greater than the mitogenic effect of serum.

The possibility of mitogenic and trophic factors in breast milk adds a new dimenion to our knowledge of gastrointesintal host defense mechanisms. However, additional studies are necessary to better understand the importance of these factors to host defense.

ACKNOWLEDGEMENTS

I wish to thank Dr. Alan Walker and Ms. Priscilla Farry for their help in preparing this manuscript.

REFERENCES

1. LEHNINGER, A.L. Enzymes: Mechanisms, structure, and regulation. In: Biochemistry. A.L. Lehninger (ed). Worth Publishers, New York, 1977, pp. 217-248.

2. HADORN, B. The exocrine pancreas. In: Paediatric Gastroenterology. C.M. Anderson, V. Burke (eds)., Blackwell Scientific Publications, Oxford, 1975, pp. 289-327.

3. MUSIC, S.I., LIBONATI, J.P., WENZEL, R.P., et al. Induced Human Cholera. Antimicrobial Agents and Chemother. 10: 462-466, 1970.

4. KRAFT, S.C., ROTHBERG, R.M., KNAYER, C.M., et al. Gastric output and circulating antibovine serum albumin in adults. Clin. Exp. Immun., 2: 321-330, 1967.

5. WALKER, W.A., WU, M., ISSELBACHER, K.J., et al. Intestinal uptake of macromolecules IV. The effect of pancreatic duct ligation on the breakdown of antigen and antigen-antibody complexes on the intestinal surface. Gastroenterology, 69: 1223-1229, 1975.

6. SAFFRAN, M., FRANCO-SAENZ, R., KONG, A. A model for the study of the oral administration of peptide hormones. Can. J. Biochem., 57: 548-553, 1979.

7. UDALL, JOHN N. Unpublished data.

8. GRAND, R.J., WATKINS, J.B., and TORTI, F.M. Development of the human gastrointestinal tract. Gastroenterology, 70: 790-810, 1976.

9. ANTONOWICZ, I. and LEBENTHAL, E. Developmental pattern of small intestinal enterokinase and dissacharidase activities in the human fetus. Gastroenterology, 72: 1299-1303, 1977.

10. ZOPPI, G., ANDREOTTI, G., and PAJNO-FERRARA, F.A., et al. Exocrine pancreas function in premature and full term neonates. Pediatr. Res., 6: 880-886, 1972.

11. LAWRENCE, G., SHANN, F., FREESTONE, D.S., et al. Prevention of necrotizing enteritis in Papua, New Guinea by active immunization. Lancet, 1: 227-230, 1979.

12. WALKER, W.A. Host defense mechanisms in the gastrointestinal tract. Pediatrics, 57: 901-916, 1976.

13. MOON, H.W. Luminal and mucosal factors of small intestine affecting pathogenic colonization. In: Secretory Diarrhea. M. Field, J.S. Fortran, and S.G. Schultz (eds). Am. Phys. Soc., Bethesda, 1980. pp. 127-139.

14. PLAUT, A.G., GORBACH, S.L., NAHAS, L., et al. Studies of intestinal microflora. III. The microbial flora of human small intestinal mucosa and fluids. Gastroenterology, 53: 868-880, 1967.

15. TENNANT, B., MALM, O.J., HOROWITZ, R.E., et al. Response of germ-free, conventional, conventionalized and E. coli-monocontaminated mice to starvation. J. Nutr., 94: 151-160, 1968.

16. NELSON, D.P. and MATA, L.J. Bacterial flora associated with human gastrointestinal mucosa. Gastroenterology, 58: 56-61, 1970.

17. FRETER, R. In vivo and in vitro antagonism of intestinal bacteria against Shigella flexneri, II. The Inhibitory Mechanism. J. Infect. Dis., 110: 38-46, 1962.

18. DUBOS, R.J. and SCHAEDLER, R.W. The effect of the intestinal flora on the growth rate of mice and on their susceptibility to experimental infections. J. Exp. Med., 111: 407-417, 1960.

19. DIXON, J.M. The fate of bacteria in small intestine. J. Path. Bact., 79: 131-140, 1960.

20. WILLIAMS, R.C., SHOWALTER, R., KERN, Jr., F. In vivo effect of bile salts and cholestyramine on intestinal anaerobic bacteria. Gastroenterology, 69: 483-491, 1975.

21. BURKE, V., and ANDERSON, C.M. Normal digestive and absorptive functions of the small intestine. In: Paediatric Gastroenterology. C.M. Anderson and V. Burke (eds). Blackwell Scientific Publishers, Oxford, 1975, pp. 125-165.

22. LESTER, R. Bile acid metabolism in the fetus and newborn. In: Development of Mammalian Absorptive Processes. CIBA Symp. 70, Exerpta Medica, New York, 1979, pp. 99-115.

23. OTTAWAY, C.A., ROSE, M.L. and PARROTT, D.M. The gut as an immunological system. In: Gastrointestinal Physiology III, International Review of Physiology, Vol. 19, R.K. Crane (ed). University Park Press, Baltimore, 1979, pp. 323-356.

24. PARROTT, D.M. The gut as a lymphoid organ. Clin. Gastroenterol., 5: 211-228, 1976.

25. LEFEVRE, M.E., HAMMER, R., and JOEL, D.D. Macrophages of the mammalian small intestine: A review. J. of Reticuloendothel. Soc., 26: 553-573, 1979.

26. BOCKMAN, D.E. and COOPER, M.D. Pinocytosis by epithelium associated with lymphoid follicles in the bursa of Fabricius, appendix and Peyer's patches. An electron microscopic study. Am. J. Anat., 136: 455-477, 1973.

27. KAGNOFF, M.F. Functional characteristics of Peyer's patch lymphoid cells. IV. Effect of antigen feeding on the frequency of antigen-specific B cells. J. Immunol., 118: 992-997, 1977.

28. PIERCE, N.F. and GOWANS, J.L. Cellular kinetics of the intestinal immune response to cholera toxoid in rats. J. Exp. Med., 142: 1550-1563, 1975.

29. OWEN, R.L. Sequential uptake of horseradish peroxidase by lymphoid follicle epithelium of Peyer's patches in the normal unobstructed mouse intestine: An ultrastructure study. Gastroenterology, 72: 440-451, 1977.

30. OWEN, R.L. and JONES, A.L. Epithelial cell specialization within human Peyer's patches: An ultrastructural study of intestinal lymphoid follicles. Gastroenterology, 66: 189-203, 1974.

31. WOLF, J.L. RUBIN, D.H., FINBERG, R, et al. Intestinal M-cells: A pathway for entry of reovirus into the host. Science, 212: 471-472, 1981.

32. OGRA, P.L. Ontogeny of the local immune system. Pediatrics, (Host Defense Supplement), 64: 765-774, 1979.

33. CHANDRA, R.K. Immunological aspects of human milk. Nutr. Rev., 36: 265-272, 1978.

34. KLEINMAN, R.E. and WALKER, W.A. The enteromammary immune system. An important new concept in breast milk host defense. Dig. Dis. and Sci., 24: 876-882, 1979.

35. HEREMANS, J.F. Immunoglobulin A. In: The Antigens, Vol. II, M. Sela (ed)., Academic Press, New York, 1974, pp. 365-522.

36. STEELE, E.J., CHAICUMPA, W. and ROWLEY, D. Further evidence for cross-linking as a protective factor in experimental cholera: Properties of antibody fragments. J. Infect. Dis., 132: 175-180, 1975.

37. WRIGHT, R. Normal immune responses in the gut and immunodeficiency disorders. In: Immunology of Gastrointestnal and Liver Disease. J. Tuck (ed). Edward Arnold Publ., London, 1977, pp. 1-15.

38. WILLIAMS, R.C. and GIBBONS, R.J. Inhibition of bacterial adherence by secretory immunoglobulin A: A mechanism of antigen disposal. Science, 177: 697-699, 1972.

39. ROGERS, H.J. and SYNGE, C. Bacteriostatic effect of human milk on E. coli: The role of IgA. Immunology, 34: 19-28, 1978.

40. SAVILAHTI, E. IgA deficiency in children: Immunoglobulin-containing cells in the intestinal mucosa, immunoglobulins in secretions and serum IgA levels. Clin. Exp. Immunol., 13: 395-406, 1973.

41. BRANDTZAEG, P. Characteristics of Sc-Ig complexes formed in vitro. In: Advances in Expermental Medicine and Biology. J. Mestecky, and A. Lawton (eds). Vol 45., Plenum Press, New York, 1974, pp. 87-97.

42. ALLANSMITH, M., McCLELLAN, B.H., BUTTERWORTH, M. et al. The development of immunoglobulin levels in man. J. Pediatr. 72: 276-290, 1968.

43. SELNER, J.C., MERRILL, D.A. and CLAMAN, H.N. Salivary immunoglobulins and albumin: Development during the newborn period. J. Pediatr. 72: 685-689, 1968.

44. HANEBERG, B., and AARSKOG, D. Human faecal immunoglobulins in healthy infants and children, and in some diseases affecting the intestinal tract or the immune system. Clin. Exp. Immunol., 22: 210-222, 1975.

45. BURGIO, G.R., LANZAVECCHIA, A., PLEBANI, A., et al. Ontogeny of secretory immunity: Levels of secretory IgA and natural antibodies in saliva. Pediatr. Res., 14: 1111-1114, 1980.

46. PERKKIO, M. and SAVILAHTI, E. Time of appearance of immunoglobulin-containing cells in the mucosa of the neonatal intestine. Pediatr. Res., 14: 953-955, 1980.

47. MATTHEW, D.J., NORMAN, A.P., TAYLOR, B., et al. Prevention of eczema. Lancet, 1: 321-324, 1977.

48. SOOTHILL, J.F., STOKES, C.R., TURNER, M.W., et al. Predisposing factors and the development of reaginic allergy in infancy. Clin. Allergy, 6:305-319, 1976.

49. LABROOY, J.T. and SHEARMAN, D.J. Antibody in the defenses of the gut. In: Topics in Gastroenterology, S.C. Truelove and C.P. Willoughby (eds)., Blackwell Scientific Publications, Oxford, 1979, pp. 177-191.

50. RIEGER, C.H.L., ROTHBERG, R.M. Development of the capacity to produce specific antibody to an ingested food antigen in the premature infant. J. Pediat., 87: 515-518, 1975.

51. ABRAMS, G.D. Effects of the normal flora on the host defenses against microbial invasion, Adv. Exp. Med. Biol., 3: 197-206, 1969.

52. CRABBE, P.A., NASH, D.R., BAZIN, H., et al. Immunohistochemical observations on lymphoid tissues from conventional and germ free mice. Lab. Invest., 22: 448-457, 1970.

53. FERGUSON, A. and PARROTT, D.M.V. The effect of antigen deprivation on thymus-dependent and thymus-independent lymphocytes in the small intestine of the mouse. Clin. Exp. Immunol., 12: 477-488, 1972.

54. POLLARD, M. and SHARON, N. Responses of Peyer's patches in germ free mice to antigenic stimulation. Infect. and Immun., 2: 96-100, 1970.

55. PITTARD III, W.B. and BILL, K. Immunoregulation by breast milk cells, Cell Immunol., 42: 437-441, 1979.

56. PITTARD III, W.B. and BILL, K. Differentiation of cord blood lymphocytes into IgA-producing cells in response to breast milk stimulatory factor. Clin. Immunol. Immunopathol., 13: 430-434, 1979.

57. IYENGAR, L. and SELVARAJ, R.J. Intestinal absorption of immunoglobulins by newborn infants. Arch. Dis. Child, 47: 411-414, 1972.

58. OGRA, S.S., WEINTRAUB, D. and OGRA, P.L. Immunologic aspects of human colostrum and milk. III. Fate and absorption of cellular and soluble components in the gastrointestinal tract of the newborn. J. Immunol., 119: 245-248, 1977.

59. HILPERT, H.., GERBER, H., AMSTER, H., et al. Bovine milk immunoglobulins (Ig), their possible utilization in industrially prepared infants' milk formulae, In: Food and Immunology, L. Hambraeus, L.A. Hanson and H. McFarlane (eds)., Almquist and Wiksell International, Stockholm pp. 182-196, 1977.

60. FORSTNER, J.F. Intestinal mucins in health and disease. Digestion, 17: 234-263, 1978.

61. FILIPE, M.I. Mucins in the human gastrointestinal epithelium: A review. Invest. Cell Pathol., 2: 195-216, 1979.

62. EDWARDS, P.A. Is mucus a selective barrier to macromolecules? Br. Med. Bull., 34:55-56, 1978.

63. TOMASI, Jr., T.B. Secretory Immunoglobulins. New Engl. J. Med., 287: 500-506, 1972.

64. SCHRAGER, J. The chemical composition and function of gastrointestinal mucus, Gut, 11: 450-456, 1970.

65. LIU, Y.S.V., LOW, T.L.K., INFANTE, A., et al. Complete covalent structure of a human IgA1 immunoglobulin, Science, 193: 1017-1020, 1976.

66. NIMMERFALL, F. and ROSENTHALER, J. Significance of the goblet-cell mucin layer, the outer most luminal barrier to passage through the gut wall. Biochem. Biophys. Res. Commun., 94: 960-966, 1980.

67. KEUSCH, G.T., GRADY, G.F., TAKEUCHI, A., et al. The pathogenesis of shigella diarrhea. II. Enterotoxin-induced acute enteritis in the rabbit ileum. J. Infect. Dis., 126: 92-95, 1972.

68. SHEAHAN, D.G., JERVIS, H.R., TAKEUCHI, A., et al. The effect of staphlococcal enterotoxin on the epithelial mucosubstances of the small intestine of Rhesus monkeys. Am. J. Pathol., 60: 1-18, 1970.

69. KLIPSTEIN, F.A. and SCHENK, E.A. Enterotoxigenic intestinal bacteria in tropical sprue, II. Effect of the bacteria and their enterotoxins on intestinal structure. Gastroenterology, 68: 642-655, 1975.

70. SHERR, H.P., MERTENS, R.B., and BROOCK, R. Cholera toxin-induced glycoprotein secretion in rabbit small intestine. Gastroenterology, 77: 18-25, 1979.

71. STROMBECK, D.R. and HARROLD, D. Binding of cholera toxin to mucins and inhibition by gastric mucin. Infect. and Immun., 10: 1266-1272, 1974.

72. HARRIES, J.T. Meconium in health and disease. Br. Med. Bull., 34: 75-78, 1978.

73. ANTONOWICZ, I. and SHWACHMAN, H. Meconium in health and in disease. Adv. in Ped., 26: 275-310, 1979.

74. LEECE , J.G. and BROUGHTON, C.W. Cessation of uptake of macromolecules by neonatal guinea pig, hamster and rabbit intestinal epithelium (closure) and transport into blood. J. Nutr., 103: 744-750, 1973.

75. UDALL, J.N., PANG, K., FRITZE, L., et al. Development of gastrointestinal mucosal barrier. I. The effect of age on intestinal permeability to macromolecules. Pediatr. Res., 15: 241-244, 1981.

76. FILIPE, M.I., FENGER, C. Histochemical characteristics of the mucins of the small intestine. A comparative study of normal benign epithelial tumors and carcinoma. Histochemical Journal, 11: 277-287, 1979.

77. ISSELBACHER, K.J. The intestinal cell surface: some properties of normal undifferentiated and malignant cells. Ann. Int. Med. 81: 681-686, 1974.

78. LEV, R. A histochmical study of glycogen and mucin in developing human foetal epithelia. Histochemical Journal, 1: 152-165, 1968.

79. LEV, R., SIEGEL, H.I., BARTMAN, J. Histochemical studies of developing fetal small intestine. Histochemie, 29: 103-119, 1972.

80. CARR, I. The Fixed Macrophage. In: The Macrophage, A Review of Ultrastructure and Function. Academic Press, London, 1973, pp. 20-40.

81. JOEL, D.D., LAISSUE, J.A. and LEFEVRE, M.E. Distribution and fate of ingested carbon particles in mice. J. Reticuloendothel. Soc., 24: 477-487, 1978.

82. LEFEVRE , M.E., OLIVO, R. and JOEL, D.D. Accumulation of latex particles in Peyer's patches and their subsequent appearance in villi and mesenteric lymph nodes. Proc. Soc. Exp. Biol. Med., 159: 298-302, 1978.

83. LEFEVRE , M.E., VANDERHOFF, J.W., LAISSUE, J.A., et al. Accumulation of 2-um latex particles in mouse Peyer's patches during chronic latex feeding. Experientia, 34: 120-122, 1978.

84. SOBHON, P. The light and electron microscopic studies of Peyer's patches in non germ-free adult mice. J. Morph., 135: 457-482, 1971.

85. WAHL, L.M., WAHL, S.M., MERGENHAGEN, S.E. et al. Collagenase production by endotoxin-activated macrophages. Proc. Nat. Acad. Sci., 71: 3598-3601, 1974.

86. DAVIES, P. and ALLISON, A.C. Secretion of macrophage enzumes in relation to the pathogenesis of chronic inflammation. In: Immunobiology of the Macrophage, D.S. Nelson (ed), Academic Press, New York, 1976, pp. 428-461.

87. ABRAHAM, R., FABIAN, R.J., GOLBERG, L., et al. Role of lysosomes in carrageenan-induced cecal ulceration. Gastroenterology, 67: 1169-1181, 1974.

88. KIRSNER, J.B. Some observations of familial inflammatory bowel disease. In: The Management of Crohn's Disease. I.T. Weterman, A.S. Pena and C.C. Booth (eds)., Exerpta Media, Amsterdam, 1976, pp. 51-56.

89. WALL, A.J., DOUGLAS, A.P., BOOTH, C.C., et al. Response of jejunal mucosa in adult coeliac disease to oral prednisolone. Gut., 11 7-14, 1970.

90. KLASS, H.J. and NEALE, G. Serum and faecal lysozyme in inflammatory bowel disease. Gut, 19: 233-239, 1978.

91. MEYER, K., GELLHORN, A., PRUDDEN, J.F., et al. Lysozyme activity in ulcerative alimentary disease II. Lysozyme activity in chronic ulcerative colitis. Am. J. Med., 5: 496-502, 1948.

92. STENSON, W.F. and PARKER, C.W. Prostaglandins, macrophages, and immunity. J. of Immunol., 125: 1-5, 1980.

93. FRIEDMAN, W.F. and FITZPATRICK, K.M. Effects of prostaglandins, thromboxanes, and inhibitors of their synthesis on renal and gastrointestinal function in the newborn period. Semin. Perinatol. 4: 143-156, 1980.

94. PESKAR, B.M. On the synthesis of prostaglandins by human gastric mucosa and its modification by drugs. Biochim. Biophys. Acta., 487: 307-314, 1977.

95. ROBERT, A. Prostaglandins and the digestive system. In: The Prostaglandins, P.W. Ramwell (ed)., Plenum Press, 1977, pp. 225-266.

96. BRAY, M.A., GORDON, D., and MORLEY, J. Prostaglandins as regulators in cellular immunity. Prostaglandin Med., 1: 183-199, 1978.

97. SATTERWHITE, T.K., EVANS, D.G., DUPONT, H.L., et al. Role of Escherichia coli colonisation factor antigen in acute diarroea. Lancet, 2: 181-184, 1978.

98. EVANS, D.G., SATTERWHITE, T.K., EVANS Jr., D.J., et al. Differences in serological responses and excretion patterns of volunteers challenged with enterotoxigenic Escherichia coli with and without colonization factor antigen. Infect. and Immun., 19: 883-888, 1978.

99. EDEN, C.S. Attachment of Escherichia coli to human urinary tract epithelial cells. An in vitro test system applied in the study of urinary tract infections. Scand J Infect Dis (Suppl) 15: 1-69, 1978.

100. SMITH, H.W., and HUGGINS, M.B. The influence of plasmid-determined and other characteristics of enteropathogenic Escherichia coli on their ability to proliferate in the alimentary tract of piglets, calves and lambs. J. Med. Microbiol., 11: 471-492, 1978.

101. QUARONI, A., KIRSCH, K., HERSCOVICS, A., et al. Surface-membrane biogenesis in rat intestinal epithelial cells at different stages of maturation. Biochem. J., 192: 133-144, 1980.

102. RAUL, F., SIMON, P., KEDINGER, M., et al. Intestinal enzymes activities in isolated villus and crypt cells during postnatal development of the rat. Cell Tiss. Res., 176: 167-178, 1977.

103. DEBOTH , N.J., VAN DER KAMP, A.W. and VAN DONGEN, J.M. The influence of changing crypt cell kinetics on functional differentiation in the small intestine of the rat: Nucleotide and Protein Synthesis. Differentiation, 4: 175-182, 1975.

104. LOJDA, Z. Cytochemistry of enterocytes and of other cells in the mucous membrane of the small intestine. In: Biomembranes (vol. 4A) Intestinal Absorption. D.H. Symth (ed.), Plenum Press, London, 1974, pp. 43-122.

105. ETZLER, M.E. and BRANSTRATOR, M.L. Cell surface components of intestinal cells and their relationship to cellular differentiation. In: Development of Mammalian Absorptive Process, Ciba Foundation Symposium 70. Excerpta Medica, Amsterdam, 1979, pp. 51-68.

106. TOOFANIAN, F., KIDDER, D.E. and HILL, F.W. The postnatal development of intestinal dissacharidases in the calf. Res. Vet. Sci., 16: 382-392, 1974.

107. BRESSON, J.L., PANG, K., UDALL, J. et al. Evidence for the increased enterotoxin binding to newborn microvillus membranes: A possible explanation for enhanced toxigenic diarrhea in infancy. Gastroenterology, (Abs.), 78: 1145, 1980.

108. KALINER, M.A. Is a mast cell a mast cell a mast cell? J. of Allergy and Clin. Immun., 66: 1-4, 1980.

109. MILLER, H.R.P. and JARRETT, W.F.H. Immune reactions in mucous membranes. I. Intestinal mast cell response during helminth expulsion in the rat. Immunology, 20: 277-288, 1971.

110. ENERBACK, L. and LUNDIN, P.M. Ultrastructure of mucosal mast cells in normal and compound 48/80-treated rats. Cell Tissue Res., 150: 95-105, 1974.

111. ENERBACK, L. Mast cells in rat gastrointestinal mucosa II. Dye-binding and metachromatic properties. Acta Path et Microbiol. Scandinav. 66: 303-312, 1966.

112. MAYRHOFER, G., BAZIN, H. and GOWANS, J.L. Nature of cells binding anti-IgE in rats immunized with Nippostrongylus brasiliensis: IgE synthesis in regional nodes and concentration in mucosal mast cells. Eur. J. Immunol., 6: 537-545, 1971.

113. KELLER, R. Nippostrongylus brasiliensis in the rat: Failure to relate intestinal histamine and mast cell levels with worm expulsion. Parasitology, 63: 473-481, 1971.

114. MAYRHOFER, G. and FISHER, R. Mast cells in severely T-cell depleted rats and the response to infestation with Nippostrongylus brasiliensis. Immunology, 37: 145-155, 1979.

115. GATES, G.A., HENLEY, K.S., POLLARD, H.M. et al. The cell population of the human liver. J. Lab. Clin. Med., 57: 182-184, 1961.

116. CANTOR, H.M. and DUMONT, A.E. Hepatic suppression of sensitization to antigen absorbed into the portal system. Nature (London), 215: 744-745, 1967.

117. NAGURA, H., SMITH, P.D., and NAKANE, P.K. IgA in human bile and liver. J. of Immunol., 126: 587-595, 1981.

118. PEPPARD, J., ORLANS, E., and PAYNE, A.W.R. The elimination of circulating complexes containing polymeric IgA by excretion in the bile. Immunology 42: 83-89, 1981.

119. SOCKEN, D.J., SIMMS E.S., NAGY, B.R. Secretory component-dependent hepatic transport of IgA antibody-antigen complexes. J. of Immunol., 127: 316-319, 1981.

120. RUSSELL, M.W., BROWN, T.A., MESTECKY, J. Role of serum IgA. Hepatobiliary transport of circulating antigens. J. Exp. Med., 153: 968-976, 1981.

121. GRULEE, C.G., SANFORD, H.N. and HERRON, P.H. Breast and artificial feeding: Influence on morbidity and mortality of twenty thousand infants. JAMA, 103: 735-739, 1934.

122. GRULLEE, C.G., SANFORD, H.N. and SCHWARTZ, H. Breast and artificially-fed infants: A study of the age incidence in the morbidity and mortality in twenty thousand cases. JAMA, 104: 1986-1988, 1935.

123. GRULEE, C.G. and SANFORD, H.N. The influence of breast and artificial feeding on infantile eczema. J. of Pediatr., 9: 223-225, 1936.

124. MELLANDER, O., VAHLQUIST, B. and MELLBIN, T. Breast feeding and artificial feeding; a clinical, serological, and biochemical study in 402 infants, with a survey of the literature; the Norbotten Study. Acta. Paediatr., 48 (suppl 116): 1-108, 1959.

125. ADEBONJO, F.O. Artificial vs. breast feeding: Relation to infant health in a middle class American community. Clini. Pediatr., 11: 25-29, 1972.

126. FALLOT, M.E., BOYD III, J.L. and OSKI, F.A. Breast-feeding reduces incidence of hospital admissions for infection in infants. Pediatrics, 65: 1121-1124, 1980.

127. BUTLER, J.E. Immunologic aspects of breast feeding, anti-fectious activity of breast milk. Sermin. Perinatol. 3: 255-270, 1979.

128. GERRARD, J.W. Breast-feeding: Second thoughts. Pediatrics, 54: 757-764, 1974.

129. CUNNINGHAM, A.S. Morbidity in breast-fed and artificially-fed infants. J. Pediatr., 90: 726-729, 1977.

130. WINBERG, J. and WESSNER, G. Does breast milk protect against septicaemia in the newborn? Lancet, 1: 1091-1094, 1971.

131. REID, B., SMITH, H., and FRIEDMAN, Z. Prostaglandins in human milk. Pediatrics, 66: 870-872, 1980.

132. ROBERTS, S.A., and FREED, D.L. Neonatal IgA secretion enhanced by breast feeding. Lancet, 2: 1131, 1977.

133. WIDDOWSON, E.M., COLOMBO, V.E. and ARTAVANIS, C.A. Changes in the organs of pigs in response to feeding for the first 24 hours after birth. II. The digestive tract. Biol. Neonate, 28: 272-281, 1976.

134. HEIRD, W.C. and HANSEN, I.H. Effect of colostrum on growth of intestinal mucosa, Pediatr. Res., (Abs.) 11: 406, 1977.

135. KLAGSBRUN, M. Human milk stimulates DNA synthesis and cellular proliferation in cultured fibroblasts. Proc. Natl. Acad. Sci., 75: 5057-5061, 1978.

136. KLAGSBRUN, M. Bovine colostrum supports the serum-free proliferation of epithelial cells but not of fibroblasts in long-term culture. J. Cell Biology, 84: 808-814, 1980.

NUTRITION RESEARCH, Vol. 1, pg. 419-420, 1981
0271-5317/81/040419-02$02.00/0 Printed in the USA.
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REVIEWS OF BOOKS

Textbook of Pediatric Nutrition

Robert M. Suskind, Editor. Raven Press, New York. pp 662, 1981.

There has been a rapid proliferation of books purporting to satisfy the needs of medical students and practitioners. The ideal book has yet to be published. The book under review is the compilation of papers given at a symposium organized by the Institute for Pediatric Service of the Johnson & Johnson Baby Products Company. The arbritrary division into the major sections of Infant Nutrition, Nutritional Deficiency States, Nutritional Support of the Hospitalized Child and Clinical Nutrition is not logical. Surely, the first three sections are as clinical as the last one. Many of the best known names in nutritional sciences and pediatrics appear in the contributors' list. However, most of the contributions are a half-hearted attempt at paraphrasing previously published reviews. There is overlap between some chapters, e.g. those on gastrointestinal disorders resulting in malnutrition, and the sections on nutritional support. The length of the contributions varies considerably as does their quality. Some chapters are rewarding reading, e.g. Disorders of fat-soluble vitamins, whereas others are disappointing, e.g. Food hypersensitivity, Anorexia nervosa, Immune response, and Childhood malignancy. Many of the references are wrongly cited.

The production values of the book are excellent. However, its academic content is at best average. The book is unlikely to serve the felt needs of the student and the practitioner who needs guidance on optimal nutritional practices, both preventive and therapeutic. A stronger editorial hand is essential for achieving the objective of a uniformly excellent text;this not such a book.

Nutritional Management of Genetic Disorders

Myron Winick, Editor. Wiley-Interscience, New York.pp 231, 1979.

The book is based on contributions given at the annual symposium organized at the Columbia University. The choice of the topic is very timely. The new discipline of genetics has crossed the threshold of adolescence and stepped into adulthood. Clearly, the time is opportune to examine the role of nutrition in modifying the expression of mutant genes. We cannot alter the genetic endowment but dietary changes do constitute a euphenic form of management. Disorders of proteins and amino acids, vitamins and carbohydrates are reviewed in the opening chapters; some are general in nature, whereas others are more specific, e.g. vitamin D-resistant rickets. Diabetes, genetic obesity and hyperlipidemias are briefly discussed. Some of the material contained in the book is "old" by scientific standards of today. The bibliography also

reflects dated reviews. Most of the contributors have published more extensive and superior analyses of the subjects elsewhere. The mix of basic science and clinical implications is good in some chapters, and poor in the others. A stricter editorial direction would have been welcome. Although there are some obvious rusty spots, the book brings into focus a very important theme which will become even more paramount when the remaining environmentally related diseases vanish from the health scene.

NUTRITION RESEARCH, Vol. 1, pg. 421-424, 1981
0271-5317/81/040421-04$02.00/0 Printed in the USA.
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SUMMARIES OF CONFERENCES

Workshop on Interactions of Diarrhoea and Malnutrition: Pathophysiology, Epidemiology, and Interventions

This timely workshop sponsored by the United Nations University's World Hunger Programme, the Rockefeller Foundation, the Ford Foundation, and the International Centre for Diarrheal Disease Research, Bangladesh, was convened in Bellagio, Italy, May 11-15, 1981. An international group of research scientists and health professionals deliberated the current knowledge of the interaction of diarrhea and malnutrition, focussing on the recent advances in laboratory and field diagnostic methods, nutrient requirements during diarrhoeal episodes and recovery, and the prevention and treatment of diarrhea. A list prioritized research needs was developed by one of the four Working Groups. The participants included Black, Brunser, Cash, Chandra, Chen, Greenough, Guerrant, Harkavy, Keusch, Mahalanabis, Martorell, Mata, Ayesha and Mujid Molla, Rahaman, Rohde, Rowland, Scrimshaw, Torun, Valyasevi, Warren and Wu.

An overview of the subject indicated that diarrheal disease is a major cause of morbidity and mortality in all poor and underprivileged societies. The young under 5 years of age are particularly vulnerable. Epidemiologic studies reported in the mid-60's suggested a bidirectional causal relationship, perhaps even synergistic, wherein nutritional deficiency predisposes the host to diarrhea, and conversely, diarrheal illness exerts a significant negative influence on nutritional status. These and more recent studies have highlighted the importance of infections in causing retardation of physical growth and development. Monthly diarrhea prevalence is associated with negative increments in linear growth and weight gain. Although many types of infections and parasitic diseases are detrimental to host nutrition, diarrheal disease stands out as the most significant in causing malnutrition. In many regions of the world, seasonal changes in growth velocity reflect the relative incidence of gastroenteritis.

It was recognized that many etiologic agents are responsible for childhood diarrhoea. Most studies have focussed on rotavirus, Escherichia coli, Shigella and Vibrio cholerae. The mechanisms of disease production are markedly different in each case. In the case of V. cholerae, large numbers of bacteria are necessary to escape passage through the protective barrier of gastric acid. In the small gut, the microorganisms multiply, stick to epithelial cells, and produce an enterotoxin. The alpha subunit of the toxin stimulates the adenyl cyclase enzyme system leading to profound losses of fluids and electrolytes. Thus the syndrome,which can vary in severity from mild to life threatening, does not require systemic invasion by the bacterium or its toxin. E. coli may cause invasive disease but more often, it produces symptoms via one or both toxins, heat-labile and heat-stable. The former

stimulates the adenyl cyclase system whereas the latter activates the guanyl cyclase enzyme, resulting in sodium, chloride and water shifts similar to the events in cholera. Shigella organisms are required in very small numbers to produce infection; only ten bacteria are sufficient. The clinical picture is characterized by bloody diarrhea, lethargy, meningeal and cerebral symptoms, and systemic invasion. A cytotoxin elaborated by Shigella inhibits protein synthesis. In contrast, rotavirus infects crypt cells, attracting inflammatory response, villus atrophy and malabsorption.

A major consequence of diarrhea is worsening of nutritional deficiencies. Many pathogenetic factors are important, including reduction of food intake due to poor appetite or social-cultural practices of food withholding or both. Some studies suggest that alterations in fluid and electrolyte balance are etiologically linked to the anorexia of diarrheal disease. This provides an additional argument for early and effective correction of dehydration in affected infants and children. The reduction in food intake may amount to as much as 40 percent of normal practice. It was reported that breast-fed infants may not alter their food intake during diarrhea to the same proportion as completely weaned babies. One more feather in the cap of breast feeding! The second major mechanism of malnutrition caused by diarrhea is malabsorption of both macro-nutrients and micro-nutrients. Many underlying processes may be important, including microbial overgrowth, deranged bile salt conjugation and metabolism, pancreatic insufficiency, disaccharidase dysfunction, villus atrophy and reduction in effective absorptive surface, decreased transit time, and decreased osmolality of luminal contents. The need for more definitive studies on malbsorption in each type of diarrhea was highlighted. Finally, there is evidence of direct loss of several nutrients in the gastrointestinal tract, urine, and sweat.

The Workshop participants agreed that effective intervention programmes need not await further increase in knowledge. Based on the information and technology currently available, it should be possible to mobilize resources to significantly reduce the incidence and nutritional impact of diarrheal disease. The introduction of oral rehydration therapy in the early management of acute diarrhea has drastically cut down the mortality risk. Improved calorie and protein intake during acute diarrhea may be an important therapeutic measure with beneficial results. Finally, the Workshop emphasized the need for developing immunoprophylactic products for primary prevention of infectious diarrhea.

The deliberations of the meeting culminated in the identification of research needs in the area of diarrhea-malnutrition interrelationship. Bearing in mind the limited resources that are available to poor communities most affected by these states, a set of priorities was discussed, which may need modification in the light of local conditions. Throughout the conference, the important preventive and therapeutic role of breast feeding was underscored.

The text of the background papers, discussion summaries, and reports of the four Working Groups would be published shortly by Plenum, New York.

XII International Congress of Nutrition

The large gatherings at international scientific congresses often preclude effective exchange of information. To some extent, the recently held XII International Congress of Nutrition, San Diego, August 17-21, 1981, belied such an assumption. The Congress provided a rich academic and social fare. Held under the sponsorship of the International Union of Nutritional Sciences, the meeting brought together an international community of scientists, health professionals, sociologists, anthropologists and administrators. The opening plenary session included a review of IUNS activities by NEVIN SCRIMSHAW and regional reports by CAMBELL, DUTRA DE OLIVEIRA, GABR, OISO and OMOLULU. The European representative, HAUTVAST, provided a most entertaining review of nutrition-related activities in his region via an excellent film presentation. The keynote address was given by SOEDJATMOKO who analyzed the magnitude and causes of the world hunger problem and the possible steps that are feasible to meet this challenge.

On the first day of the Congress, PRASAD and SHAH cochaired the symposium on Trace Elements in Human Nutrition. The important physiologic role of zinc was described. The various clinical conditions resulting from zinc deficiency were reviewed. It was pointed out that there are complex interactions between various trace elements; this compounds the problems of attributing effects to a single nutrient deficiency. The bioavailability of trace elements was discussed with particular reference to dietary fiber. The toxic effects of trace heavy metals were outlined. In the sumposium on Nutrition, Brain Function,and Behavior, cochaired by BROZEK and ROSSETTI-FERREIRA, the neurophysiologic basis of nutritional effects on the central nervous system structure, biochemistry and function were reviewed. The consequences of fetal iodine deficiency may be severe and permanent. The results of prospective studies of supplemented and unsupplemented groups of Mexican children with reference to school performance were presented. The assessment of cognitive performance is an important technique for determining nutrition-related alterations in C.N.S. function.

Tuesday morning symposia included one on Marginal Malnutrition moderated by BEHAR and UNDERWOOD. An excellent introductory review discussed the scope of the public health problem and the various techniques available to assess mild malnutrition. The main yardstick applied world-wide is anthropometric measurement. The limitations of this method have led to exploration of other approaches, the most promising of which may be immunocompetence. Several studies have shown the profound changes in immune responses as a direct result of moderate-severe nutritional deficiencies. It was suggested that infants and children with marginal deficits of energy, protein or single nutrient, e.g. trace elements, do exhibit detectable changes in immunocompetence which can serve as functional index of nutritional status. The influence of marginal malnutrition on menarche, fecundity and fertilization was extensively reviewed. It was pointed out that mild nutritional deficiency may have a significant impact on learning and social performance, a theme developed again by the Atwater lecturer, CRAVIOTO. Marginal malnutrition decreases work performance and has functional consequences for both industrialized and developing countries. SAI and CHAFKIN cochaired the session on The Nutrition Component of National Policy and Planning. The difficulties encountered in

assessing the nutritional needs of countries, choosing the appropriate methods for reducing the impact of malnutrition and indices for monitoring the success, both short-term and long-term, of intervention programmes were discussed. Examples were cited from various projects in Africa, Asia and the Americas.

The subject of Nutritional Status and Susceptibility to Disease was reviewed in a session organized by CHANDRA and KEUSCH. The microbiologic, host and environmental factors important in the complex biology of infectious disease were outlined. It was shown that nutritional deficiency states are associated with variable impairment of immune response, largely correctable immunodeficiency. The one exception to this is the case of the small-for-gestational age low birth birth weight infant who may continue to exhibit reduced immunocompetence and higher morbidity from infection. The mechanisms, magnitude and clinical significance of infection-induced nutrient deficiency were outlined. There was a brief review of the community level intervention programmes which can reduce the impact of both infection and malnutrition. The important but controversial data on Protein-energy Requirements and Interactions was reviewed in the symposium cochaired by YOUNG and TORUN. The physiologic control of energy intake and the interaction of total calorie ingestion and protein intake were discussed. Physical activity has an important bearing on needs.

On the third day, FAJARDO and AUSTIN moderated a session on Nutrition Intervention Programmes. The objectives, broad principles and limitations of such efforts were reviewed. It was felt that careful analysis must precede the scaling up of pilot projects to national level strategies. The active input of the community involved is important for a long-lasting impact. In promoting such interventions, the international agencies play an important catalytic role. Drug-Nutrient Interactions were reviewed by McLEAN AND ROE. Data on absorption, metabolism, excretion and body distribution were presented. Many pharmacological agents exert marked effects on nutrient needs.

Thursday morning symposium on the Fat-Soluble Vitamins was organized by BRUBACHER and OLSON. The prevalence of fat-soluble vitamin deficiencies world-wide was reviewed, particularly in relation to possible causes and interactions with other deficiency syndromes. The biochemical and metabolic role of various vitamins was discussed. HALLBERG and COOK moderated the session on Nutritio-nal Anemias. The harmful effects of iron deficiency, assessment of iron status and bioavailability of iron in foods was reviewed. Iron supplementation and fortification programmes were described. In the afternoon, SOYSA and METCOFF cochaired the symposium on Maternal, Fetal and Neonatal Nutrition. The important influence of nutrition on maternal leukocyte enzymes was described and research needs in maternal-fetal nutrition interactions were identified. BRAUDE and TEAGUE reviewed the subject of the Function of Gastrointestinal Tract in Health and Disease. The last day saw interesting presentation on Nutrition and Chronic Disease co-chaired by TASHEV and CHRISTAKIS, and Nutritional Care of Hospitalized Patients by PICOU and BUTTERWORTH.

The text of symposia presentations will be published shortly by Alan Liss, New York. A brief review of Minisymposia and selected Free Communications will appear in the next number of Nutrition Research.

NUTRITION RESEARCH, Vol. 1, pg. 425, 1981
0271-5317/81/040425-01$02.00/0 Printed in the USA.
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ANNOUNCEMENTS

Symposium on Nutrition in Gerontology and Geriatric Medicine

This conference will be held at the Bethesda Marriott Hotel, September 14-15, 1981. Organized by the American College of Nutrition, the symposium will feature lectures by MITCHELL KAMINSKY on Surgical Nutrition with Special Reference to Parenteral and Enteral Nutrition in the Geriatric Patient, REUBIN ANDRES on Critical Review of Obesity-Mortality Association, JAMES ANDERSON on Changing Concepts in the Dietary Management of Adult Onset Diabetes Mellitus, NATHAN SHOCK on the Physiology of Aging, DAPHNE ROE on Nutrition-Medication Interrelationships in the Geriatric Patient, RANJIT CHANDRA on Nutrition and Immunocompetence in the Elderly, ROBERT LINDEMAN on Mineral Metabolism in Aging and the Aged, RAYMOND HARRIS on the Role of Nutrition in the Etiology, Pathogenesis and Treatment of Cardiovascular Diseases, RICHARD RIVLIN on Nutrition and Cancer, and CHARLES GLUECK on Atherosclerosis from Cradle to Grave. In addition to the symposium on old age, the College meeting will have two afternoon sessions of free communications held under the aegis of various Councils.

For information and registration, write to the American College of Nutrition, 100 Manhattan Avenue, #1606, Union City, New Jersey 07087.

Special Subscription Rates for NUTRITION RESEARCH

In consultation with the American Institute of Nutrition and the American Society for Clinical Nutrition, a special reduced subscription rate of $20 annual for members of these bodies and $18 annual for students is available. Similar concessional rates are applicable to members of national nutrition societies of other countries. Nonmember individuals whose institutions subscribe to Nutrition Research may obtain a yearly subscription at $30.

Nominations for the William C. Rose Lecturership

The Award commemorates Dr. William C. Rose whose work at the University of Illinois, Urbana, included the discovery of threonine and the assessment of the essential nutrient requirements of the laboratory rat and of man. The Award Lecturership in Nutrition and Biochemistry emphasizes characteristics of excellence in science and personal relationships. The Award is administered by the Nutrition Foundation, to which nominations may be sent at 489 Fifth Avenue, New York, NY 10017.

NUTRITION RESEARCH, Vol. 1, pp. 427-428, 1981
0271-5317/81/050427-02$02.00/0 Printed in the USA.
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EDITORIAL

Immunocompetence in Vitamin A Deficiency

Protein-energy malnutrition has profound effects on lymphoid organs and immune responses. It is recognized that human malnutrition syndromes are not homogeneous entities. A variety of nutrient deficiencies occur together in the same individual and each may be responsible, in part, for the impaired immunocompetence observed in undernourished subjects. It is now established that zinc, iron and pyridoxine, among other elements, exert significant influence on cell-mediated immunity (1), although each probably acts through different mechanisms. Vitamin A deficiency frequently complicates protein-energy malnutrition and is the most common vitamin deficiency found world wide. It is therefore essential to examine the role of this nutrient in immune responses. Many recent studies have addressed this question.

Animals fed vitamin A-deficient diet show increased vulnerability to many infectious agents, including bacteria, viruses and parasites. There is a partial involution of the thymus, reduction in the number of mononuclear cells and decrease in DNA synthesis (2). Vitamin A deficiency results in a moderate reduction in antibody responses, particularly to those antigens which require the help of thymus-dependent T cells, such as tetanus toxoid and heterologous red blood cells (3). Serum complement levels are reduced (4). Lymphocyte stimulation response to mitogens is decreased (3), a finding confirmed and extended by Mark and his colleagues in this issue of Nutrition Research. Delayed cutaneous hypersensitivity is impaired and there is a slight reduction in the number of circulating T lymphocytes. Deprived animals have significant changes in epithelial cells, particularly the cilia. Partly as a result of these morphologic changes, the amount of secretory component seen on immunofluorescent staining of intestinal sections is reduced (5).

Many pathogenetic mechanisms may underlie the impaired cell-mediated immunity observed in vitamin A deficient individuals: Reduction in the number of responding cells, alterations in cell-surface glycoprotein receptors for mitogens and antigens, altered homing and traffic patterns of committed and stimulated lymphocytes, impaired differentiation, changes in thymic inductive factors, etc. Some of these pathways have been examined in vitamin A deficient animals. For example, there is a reduction in the number of rosette-forming T cells in children with isolated vitamin A deficiency. The cellular traffic of labelled lymphocytes has been monitored in viatmin A deficiency and found to be altered (5). On the other hand, serum thymic factor activity was found to be unchanged (6). There are marked changes in cell-surface glycoproteins of lymphocytes (5).

Vitamin A deficiency has significant effects on cell-mediated immunity and this may be responsible in part for the increased susceptibility to infection.

R. K. Chandra

1. CHANDRA, R.K. and NEWBERNE, P.M. Nutrition, Immunity and Infection: Mechanisms of Interactions. Plenum, New York, 1977.

2. KRISHNAN, S., BHUYAN, U.N., TALWAR, G.P. and RAMALINGASWAMI, V. Effect of vitamin A and protein-calorie deficiency on immune responses. Immunology 27:383-389, 1974.

3. CHANDRA, R.K. and AU, B. Single nutrient deficiency and cell-mediated immune responses. III. Vitamin A. Nutr. Res. 1:181-185, 1981.

4. MADJIT, B., SIRISINHA, S. and LAMB, A.J. The effect of vitamin A and protein deficiency on complement levels in rats. Proc. Soc. Exp. Biol. 158:92-95, 1978.

5. CHANDRA, R.K. Unpublished data.

6. CHANDRA, R.K., HERESI, G. and AU, B. Serum thymic factor activity in deficiencies of calories, zinc, vitamin A and pyridoxine. Clin. exp. Immunol. 42:332-335, 1980.

NUTRITION RESEARCH, Vol. 1, pp. 429-442, 1981
0271-5317/81/050429-14$02.00/0 Printed in the USA.
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THE CHOLESTEROLEMIC EFFECT OF SKIM MILK IN YOUNG MEN CONSUMING CONTROLLED DIETS[1,2,3]

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ABSTRACT

A 34-day metabolic study was conducted with nine healthy adult men to test the hypocholesterolemic action of skim milk (2 qt/day). Intakes of kilocalories , carbohydrate, protein, fat, cholesterol, calcium and dietary fiber were controlled, as were the ratios of polysaturated to saturated fat and animal protein to total protein in the diet. The study was comprised of three experimental periods: a high calcium control period (2600 mg/day), a low calcium control period (800 mg/day), and a high calcium skim milk period (2600 mg/day). Consumption of skim milk was associated with increases in plasma cholesterol levels, low density lipoprotein (LDL)-cholesterol and LDL-total lipid levels, and ratio of plasma cholesterol to high density lipoprotein (HDL)-cholesterol; and a decrease in HDL-cholesterol. A high dietary calcium intake was associated with a decrease in the plasma cholesterol to HDL-cholesterol ratio and an increase in triglyceride content of very low density lipoproteins. In this study, no hypocholesterolemic effect of fresh skim milk was observed when it was consumed along with a diet representative of a typical American diet pattern.

KEY WORDS: skim milk, dietary calcium, plasma cholesterol, low density lipoproteins, high density lipoproteins, hypocholesterolemic milk factor

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[1]This work was supported in part by the College of Agricultural and Life Sciences, University of Wisconsin-Madison, Hatch Project #5141, and Doyle Pharmaceutical Co., Minneapolis, MN.

[2]A preliminary report of this work has been presented. Hunsdorfer, N.K. and Marlett, J.A. Fed. Proc. 39: 649, 1980.

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INTRODUCTION

Milk and other dairy products contribute substantially to the nutritional quality of the American diet by providing significant quantities of protein of high biological value, as well as calcium, riboflavin, and vitamin B_{12} (1). Despite the nutritional density of milk, its saturated fat content has linked this foodstuff with atherogenesis (2), and reduced consumption of whole milk has been recommended (3). However, both fresh and fermented milks have been observed to decrease cholesterolemia in man. Mann and Spoerry reported that the consumption of fermented bovine milk by the Maasai tribesmen lowered serum cholesterol values (4). Subsequently, this observation was confirmed in free-living Americans who supplemented their usual diets with 2 to 4 liters of yogurt daily (5). In addition, sufficient quantities of whole milk, skim milk, and 2% lowfat milk have been reported to lower plasma cholesterol levels by 5-15% when added to normal diets (6-9). While these studies suggest that milk may contain factors that lower plasma cholesterol in man, they suffer from inadequate control of dietary intake during the period when the milk products were consumed. The present study was designed to test the hypocholesterolemic properties of skim milk while controlling intake and distribution of kilocalories, cholesterol, calcium, and dietary fiber, as well as the ratios of polyunsaturated to saturated fat and animal protein to total protein in the diet. Since high dietary calcium is reported to cause mild hypocholesterolemia (10-15), two levels of calcium were fed during the control periods to determine its possible contribution. Plasma cholesterol and lipoprotein-lipid levels were measured under these constant metabolic diet conditions.

METHODS

Subjects. Nine healthy young adult males, aged 18 to 32 years, participated in this study which had been approved by the Human Subjects Committee of the College of Agricultural and Life Sciences, University of Wisconsin-Madison. One week prior to the study, the subjects' plasma cholesterol levels were within the normal range of 150-250 mg/dl, and lipoprotein profiles, determined by agarose gel electrophoresis (Universal electrophoresis film, Corning ACI, Palo Alto, CA), were normal. Eight of the nine subjects were of appropriate weight for height; one subject weighed 9% greater than ideal. All subjects routinely consumed milk ($\geq$ 2 cups/day) as part of their usual diets.

TABLE 1

Sources of Dietary Calcium

	Experimental Period		
	I High Calcium Control	II Low Calcium Control	III High Calcium Skim Milk
Calcium sources, mg/day			
Food	498	498	309
Calcium gluconate	112	311	0
Calcium phosphate (dibasic)	2037	0	0
Skim Milk	0	0	2306
Total calcium intake, mg/day	2647	809	2615

Experimental Design. The metabolic study was 34 days long and included three experimental periods: Period I--high calcium control period (10 days), Period II--low calcium control period (10 days), and Period III--high calcium skim milk period (14 days) during which two quarts of fresh skim milk were added to the daily diet. Calcium supplements were taken with the daily food to provide the recommended dietary allowance (16) for calcium in Period II and to equalize the calcium intake of Period I with that of Period III (Table 1).

TABLE 2

Food of Daily Diet

Item	Control Periods, I, II g/day	Skim Milk Period, III g/day
Orange juice	180	125
Egg	50	50
Cornbread[a]	120	110
Butter	40	40
Jelly	15	0
Tunafish	100	60
Onion	40	40
Celery	10	10
Mayonnaise	25	25
Dill Pickle	30	30
Carrot	30	30
Whole wheat bread	56	56
Cherry muffin[a]	80	58
Ground beef	138	130
Macaroni	50	50
Tomato juice	250	250
Green pepper	30	30
Green beans	100	100
Lettuce	70	70
Cucumber	30	30
French dressing	10	10
White bread	25	13
Applesauce cake[a]	108	82
Skim milk	0	1960
7-UP	682	0

[a]During Periods I and II a total of 56.5 g/day purified casein was incorporated into these 3 quickbreads.

Diets. The diet was designed to represent a typical American diet pattern (Table 2). The intake of kilocalories and the distribution of carbohydrate, protein and fat were kept constant throughout the study, as were the intake of cholesterol, plant sterols, dietary fiber and the ratio of polyunsaturated to saturated (P:S) fats (Table 3). Also, the percent of protein from animal sources was kept constant by incorporating purified casein (Erie Casein Co., International Protein Division, Erie, PA) into the baked goods when no skim milk was consumed. The phosphorus content of the diet was 3400 mg during Period I, 1700 mg during Period II, and 2900 mg during Period III. Diet composites of the daily food were prepared prior to the study and twice during each experimental period. Blended homogenates were lyophilized, and portions of the dried diet were taken for sterol (17), dietary fiber by a modification (18) of the Van Soest Procedure (19), calcium (20), and phosphorus (21) analyses.

TABLE 3

Daily Intake of Selected Nutrients

		Control Diets (I, II)[a]	Skim Milk Diet (III)
kilocalories[b]		2985	2974
carbohydrate[b],	g	318	314
	% Kcal	42.6%	42.2%
protein[b],	g	147	149
	% Kcal	19.7%	20.1%
fat[b],	g	125	125
	% Kcal	37.7%	37.7%
% protein from animal sources[b]		78	78
P:S ratio[b]		0.64	0.63
cholesterol[c],	mg	639	640
plant sterols[c],	mg	466	442
dietary fiber[c],	g	10.2	9.2

[a]Periods I and II diets differed only in calcium content.

[b]Computed from Agriculture Handbook No. 456 (22).

[c]Determined by analysis (see text).

The skim milk was prepared in a single batch by the University of Wisconsin Dairy Plant in the Department of Food Science. Raw milk from several local farms was pooled, pasteurized, defatted and packaged the day before the milk supplementation began. The milk was not fortified with vitamin D; instead, the subjects received a vitamin D supplement (Drisdol, Winthrop Laboratories, New York, NY) providing 400 I.U. of ergocalciferol daily throughout the study. Subjects were permitted to consume double-distilled water as desired. No other foods or beverages were permitted during the study.

Due to a slight, but progressive weight gain of one subject the caloric level of his diet was lowered to 2760 kilocalories beginning on study day 20. By lowering the daily caloric intake, the diet, which was consumed during Period III, contained 139 g protein (20.1% Kcal), 116 g fat (37.8% Kcal), and 290 g carbohydrate (42.0% Kcal), 619 mg cholesterol, 2590 mg calcium, and 2825 mg phosphorus. The P:S ratio remained the same. This diet modification had no apparent effect on the plasma cholesterol and lipoprotein-lipid response of this subject.

For the week preceding the study, subjects were instructed to keep a daily food log of all foods and beverages consumed. Protein, carbohydrate, fat, calcium (22) and cholesterol (23) contents of these diets were computed. Mean daily intakes of these nutrients by the subjects were similar to the levels in the experimental diets: kilocalories: 3027 ± 967 (S.D.); % protein: 16.0 ± 3.6%; % fat: 38.1 ± 6.0%; % carbohydrate: 43.0 ± 5.7%; cholesterol: 512 ± 131 mg; and calcium: 1490 ± 548 mg.

Blood Collection and Analyses. Cholesterol determinations (24) were performed on EDTA-plasma obtained from fasting subjects seven days before the study commenced, on the first day of the study and during the study at five-day intervals. Initial cholesterol values for study day 0 were calculated as the mean of the values determined seven days prior to the study and the first day of the study. Plasma lipoproteins were isolated and analyzed on study days 10, 20, and 34.

A one-step ultracentrifugation procedure was used to separate plasma lipoproteins. For each plasma sample, one aliquot was spun at its native density of 1.006, a second at density=1.063 and a third at density=1.21. Density adjustments were made by the addition of a concentrated cesium chloride solution. These samples were centrifuged simultaneously in a Beckman L2-65B ultracentrifuge in a type 35 rotor fitted with 7-hole aluminum adapter (Beckman Instruments, Palo Alto, CA). Centrifugation was carried out at 15° C, 30,000 rpm, for 20 hours. Lipoproteins were recovered by careful manual aspiration of the supernatant and infranatant, rinsing tubes with 0.9% NaCl. The supernatant of the d=1.006 tube contained very low density lipoprotein (VLDL); the infranatant, low density lipoproteins (LDL) plus the high density lipoproteins (HDL). The supernatant of the d=1.063 tube contained VLDL + LDL; the infranatant, HDL. All three lipoprotein classes were present in the supernatant of the d=1.21 tube. Recovery was monitored by cholesterol determination of all lipoprotein fractions and averaged 88.0 ± 7.8% (S.D.).

Total cholesterol determinations (24) were performed directly on lipoprotein fractions. A total lipid extract (25) of lipoprotein fractions was chromatographed on Silica Gel H using a two-step solvent system (26) for separation of free and esterified cholesterol. Recovery of total cholesterol following TLC averaged 94.I ± 4.0%. Phospholipids were eluted from the Silica Gel H (27) and measured by the method of Bartlett (28). Triglyceride content was determined on all the supernatant lipoprotein fractions by the colorimetric method of Fletcher (29) using reagent preparations suggested by Foster and Dunn (30).

Statistical Analysis. Plasma cholesterol levels and lipoprotein-lipid data were analyzed statistically by multiple regression analysis (31). The influence of the presence or absence of high dietary calcium or skim milk was tested on the dependent variables: plasma cholesterol levels, lipoprotein-cholesterol levels, plasma cholesterol to HDL-cholesterol ratio, and lipoprotein-lipid composition. The equation took the form:

$$\text{dependent variable} = b_0 + b_1(\text{high calcium}) + b_2(\text{skim milk}) + b_3(\text{covariate}).$$

Values for the dependent variable at study day 20, the end of the low calcium control period, were used as covariate. This procedure corrected for individual responses to the experimental diets. All results are expressed as mean ± SEM.

RESULTS

Effect of controlled diets on plasma cholesterol concentrations.

The mean cholesterol concentration at the start of the study was 207 ± 10 mg/dl. After ten days of the high calcium control diet, this value had decreased insignificantly by 8% to 191 ± 11 mg/dl. Individually, seven of the nine subjects responded to the high calcium control diet with a decrease in plasma cholesterol level (Figure 1). Mean plasma cholesterol level remained near this level during the low calcium control period with values of 192 ± 13 mg/dl and 194 ± 16 mg/dl, determined after 5 and 10 days of this diet, respectively.

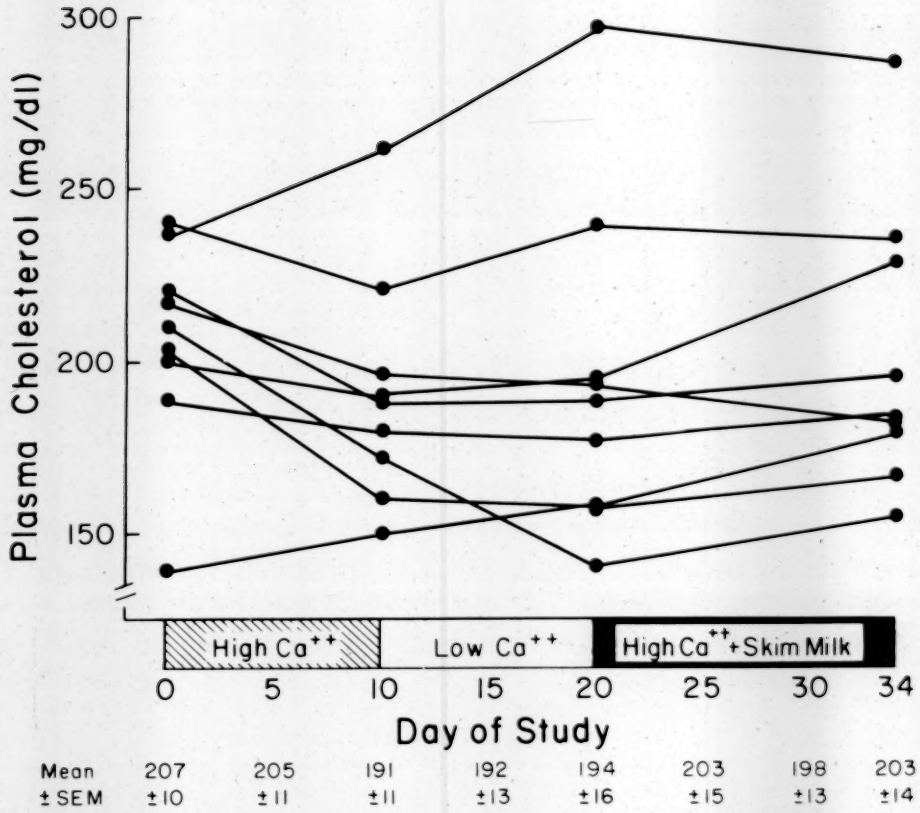

FIG. 1

Individual Responses in Plasma Cholesterol Levels to Controlled Diets With or Without Skim Milk. By multiple regression analysis, the presence of skim milk in the diet (day 34) significantly increased ($p < 0.05$) mean plasma cholesterol levels compared to either the high (day 10) or low (day 20) calcium control periods.

Incorporation of skim milk into the diet did not produce a further decline in plasma cholesterol concentrations (Figure 1). In fact, mean plasma cholesterol levels rose to 203 ± 15 mg/dl after 5 days, 198 ± 13 mg/dl after 9 days, and finally to 203 ± 14 mg/dl after 14 days of skim milk consumption. This represents a modest 5% increase above mean plasma cholesterol levels of the control periods I and II, but it is statistically significant ($p < 0.05$). When compared to the high calcium control period (I), seven of the nine subjects responded to the presence of skim milk in the daily diet with increases in plasma cholesterol levels (Figure 1).

Lipoprotein-cholesterol. Low density lipoprotein-cholesterol levels increased significantly ($p < 0.001$) by the end of the skim milk period, compared to both control periods (Figure 2); increases were observed in seven of the nine subjects. Conversely, HDL-cholesterol levels decreased significantly ($p < 0.05$) with skim milk supplementation despite an overall increase in plasma total cholesterol. The VLDL-cholesterol levels did not change significantly during the course of the study (Figure 2).

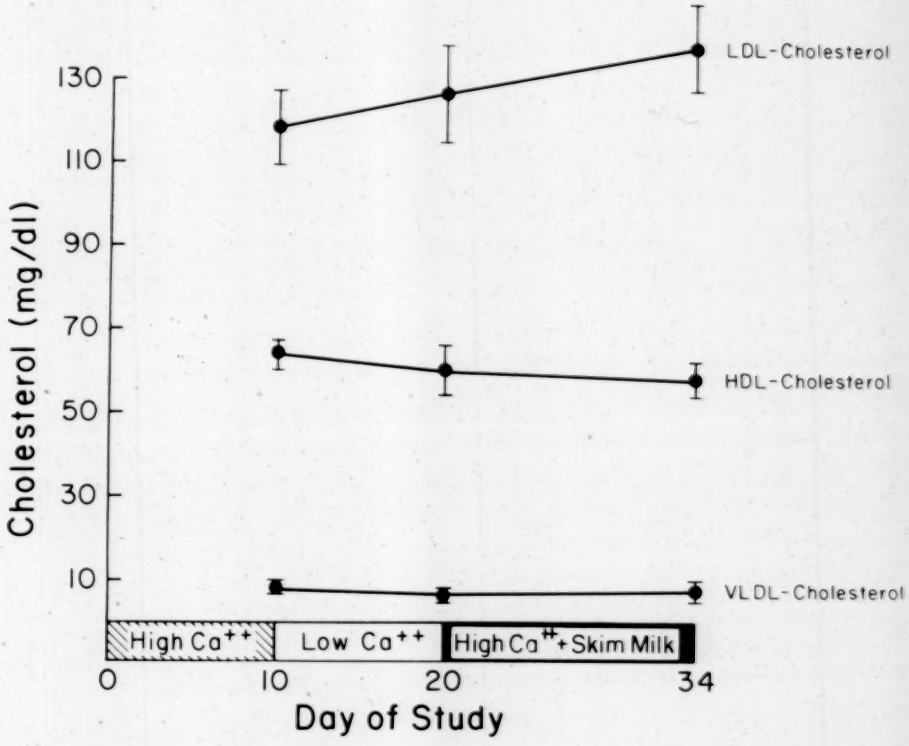

FIG. 2

Effects of Calcium or Skim Milk Supplementation on Plasma Lipoprotein-Cholesterol Levels. Each point represents the mean of nine subjects, vertical bars are the SEM. Multiple regression analysis showed that the presence of skim milk in the diet significantly increased ($p < 0.001$) LDL-cholesterol and significantly decreased ($p < 0.05$) HDL-cholesterol.

The ratio of plasma total cholesterol to HDL-cholesterol was lowered significantly ($p < 0.05$) in Period I (Figure 3). On the other hand, a highly significant increase ($p < 0.001$) was observed in the ratio with skim milk in the diet in Period III (Figure 3). The higher ratio associated with the presence of skim milk in Period III occurred despite the presence of high dietary calcium which appeared to exert an opposing effect on the ratio during Period I.

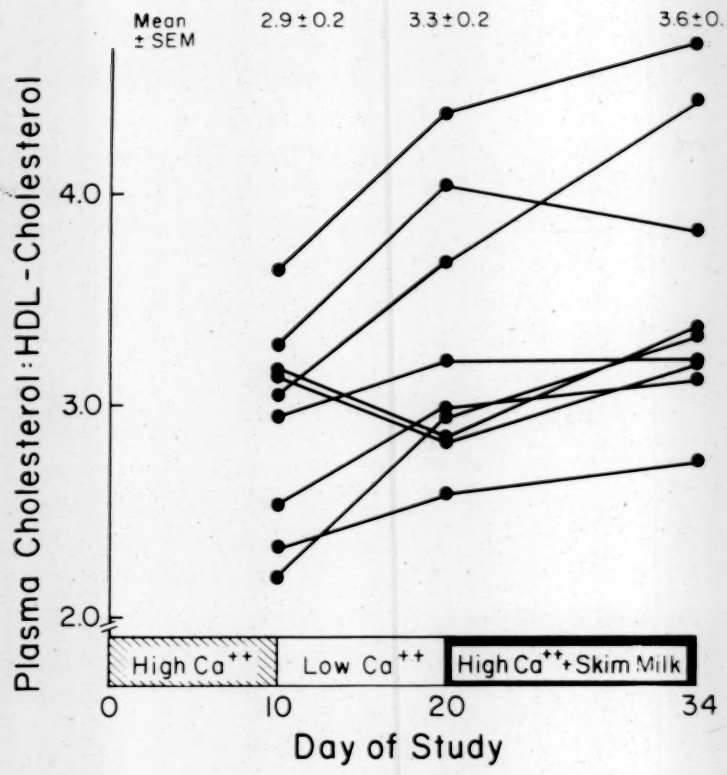

FIG. 3

Changes in Ratios of Plasma Cholesterol: HDL-Cholesterol during Calcium or Skim Milk Supplementation. Each group of three points joined by a straight line represents an individual response in the ratio to high calcium control diet, low calcium control diet, and high calcium skim milk diet. As verified by multiple regression analysis, high dietary calcium levels decreased the mean ratio significantly ($p < 0.05$) and skim milk increased the mean ratio significantly ($p < 0.001$).

Lipoprotein-lipid composition. VLDL. Triglyceride content of VLDL increased significantly ($p < 0.05$) in the presence of high dietary calcium levels (Table 4). This increase occurred whether calcium was supplemented in the form of calcium gluconate and phosphate or skim milk. Similarly, the VLDL-total lipid concentrations tended to be higher when the calcium level of the diet was high (Table 4).

LDL. The increase in LDL-cholesterol observed during skim milk supplementation was accompanied by a significant increase in LDL-total lipid ($p < 0.05$). When skim milk was present in the diet, there was a tendency for the lipoprotein core components, triglyceride and cholesterol ester, to decrease and the surface components, phospholipid and free cholesterol, to increase (Table 4). Free cholesterol in LDL was significantly increased ($p < 0.005$) by skim milk supplementation.

TABLE 4

Lipid Composition of Plasma Lipoproteins[a]

Diet Period	Total Lipid mg/dl	Triglyceride	Phospholipid	Cholesterol Ester	Free Cholesterol
		% of Total Lipid			
Very low density lipoproteins					
High Ca^{++} Control	55.5 ± 10.2	66.4 ± 5.3[b]	18.6 ± 4.5	7.4 ± 1.2	7.7 ± 0.7
Low Ca^{++} Control	41.5 ± 6.1	55.3 ± 2.6	27.3 ± 2.8	8.4 ± 0.4	9.1 ± 1.1
High Ca^{++} Skim Milk	53.1 ± 11.2	72.2 ± 2.5[b]	14.0 ± 1.9	6.1 ± 0.8	6.8 ± 0.6
Low density lipoproteins					
High Ca^{++} Control	194.6 ± 15.1	12.0 ± 1.4	26.8 ± 0.8	42.3 ± 1.0	19.0 ± 0.8
Low Ca^{++} Control	211.0 ± 18.2	14.0 ± 2.6	25.9 ± 0.8	39.6 ± 1.5	20.4 ± 0.8
High Ca^{++} Skim Milk	220.8 ± 15.3[d]	9.9 ± 1.5	27.9 ± 1.5	39.2 ± 1.0	23.0 ± 0.8[c]
High density lipoproteins					
High Ca^{++} Control	162.6 ± 8.6	10.5 ± 1.8	49.7 ± 1.6	28.2 ± 1.6	11.6 ± 0.6
Low Ca^{++} Control	158.0 ± 13.2	8.3 ± 1.0	53.0 ± 3.1	27.0 ± 1.5	11.7 ± 0.9
High Ca^{++} Skim Milk	146.4 ± 9.4	9.4 ± 1.0	50.8 ± 1.2	27.9 ± 1.1	11.9 ± 0.5

[a]Mean ± SEM

[b]Significant effect associated with presence of high diet calcium ($p < 0.05$)

[c]Significant effect associated with presence of skim milk ($p < 0.005$)

[d]Significant effect associated with presence of skim milk ($p < 0.05$)

HDL. An insignificant decrease in HDL-total lipid was observed during skim milk supplementation, but the lipid composition of the HDL was similar through all diet periods (Table 4). These data suggest that there were fewer numbers of HDL circulating during the period of skim milk consumption.

DISCUSSION

The results of this study suggest that fresh skim milk does not contain a hypocholesterolemic factor that is biologically active under dietary conditions typical of the U.S. population. In fact, when the plasma cholesterol responses to the high calcium periods are compared, skim milk increased plasma cholesterol levels in seven of nine subjects by an average of 10.6 ± 2.1%. The remaining two subjects had reduced cholesterol levels of 7% and 10%. Certain studies (11,12) examining the influence of diet modification on plasma cholesterol levels have found that the % change in cholesterolemia is dependent on initial plasma cholesterol levels. The magnitude of changes we observed with skim milk ingestion were not related to initial plasma cholesterol levels of the subjects.

Previous studies examining the influence of fresh milk consumption on plasma cholesterol levels have yielded inconsistent results; failure to rigorously control intake may be a primary contributor to this inconsistency. In agreement with our study, Hussi, et al. (32) observed no significant hypocholesterolemic effect of skim milk ingestion (2.7 liters/day for 3 weeks) by healthy male prisoners. This study is the only one reported to date that has carefully controlled energy, protein, carbohydrate, fat, cholesterol and fiber intakes. Mitchell and coworkers (10) also failed to observe a decrease in plasma cholesterol levels of elderly hypercholesterolemic subjects receiving 1.5 pints of reconstituted nonfat dry milk daily over a period of four weeks; in fact, plasma cholesterol concentrations also tended to increase in their study. Although their experiment was not conducted through a metabolic kitchen, as was ours, they did have subjects consuming "fixed quantities of calcium, fat and sterols." Other investigators (5,9) reported no change in serum cholesterol levels when 2 liters of fresh whole milk were consumed daily for 12-21 days while subjects maintained normal eating habits. Undoubtedly, the milk supplement was a substitution, not an addition to the diet, and a redistribution of energy sources occurred. In fact, with the aid of diet records, Rossouw, et al. (9) found that fat and cholesterol intakes increased significantly on the whole milk diet.

Hepner, et al. (7) found that the addition of 1.5 pints/day of 2% lowfat milk or yogurt did not change mean daily intakes of fat, cholesterol and fiber significantly. But, Hepner and associates were unable to record consistent cholesterolemic responses when 1.5 pints of fresh 2% lowfat milk was consumed by 17 adults. In their experiment, which used a cross-over design, lowfat milk had no effect on plasma cholesterol concentrations in subjects taking milk during the last four weeks of the study, whereas it lowered plasma cholesterol by an average of 4% in subjects taking milk during the first four weeks of the study. A second separate study examined plasma cholesterol levels of ten adults who supplemented their usual diets with 1.5 pints of 2% lowfat milk/day for 12 weeks; at the end, mean plasma cholesterol level was 6% lower than the initial level (7). Other laboratories (6,8,9) have reported plasma cholesterol reductions of 9-15% over 2-3 weeks when 2 quarts of skim milk were consumed daily. In the studies of Howard and Marks (6,8) no information is available regarding what kinds of foods were replaced by the skim milk. In the study of Rossouw, et al. (9), skim milk consumption led to increased protein and calcium intakes. Certainly great disparity exists in the reports of the cholesterolemic effect of fresh milk; perhaps more complete information on the composition of the diets consumed with the milk could explain some of the variability in plasma cholesterol response.

The hypocholesterolemic activity of fresh milk products has been attributed to several substances in milks; of these, calcium possesses a modest hypocholesterolemic action in man (10-15). Ingestion of a calcium supplement, equivalent to 1-2 g calcium, along with a typical diet containing 300-900 mg calcium, leads to 3-25% reduction in plasma cholesterol levels (10-15). In general, the effect is more pronounced in hypercholesterolemic subjects (11,12) and has been sustained for a year or longer (13,14). In healthy subjects with normal cholesterol levels, a hypocholesterolemic response of 4-6% has been observed (11,15) which corresponds to the 8% decrease in plasma cholesterol concentrations observed in the present study during the high calcium control period. Our results could be attributable to increased calcium intake from the subjects' usual levels of approximately 1500 mg/day to 2600 mg/day, but the fact they maintained similar cholesterol levels at the lower calcium intake of 800 mg/day reduces the possibility that high calcium intake per se lowers cholesterol levels. Furthermore, our statistical model permitted separation of the influence of skim milk and the dietary calcium level, and the presence of high calcium levels in the diet showed no significant influence on plasma cholesterol levels ($p = 0.57$). Alternately, a shift to a rigorously controlled daily intake which represents a significant change in life-style, might have had an impact on plasma cholesterol levels. Even the administration of daily placebo tablets for four weeks has resulted in a significant decrease ($p < 0.01$) in plasma cholesterol levels (13).

The decrease in HDL-cholesterol associated with skim milk ingestion in the present study was surprising, since HDL has been reported to be fairly resistant to dietary perturbations (33). Nonetheless, our findings concur with those of Rossouw, et al. (9). Decreases in HDL-cholesterol accompanied by elevated triglyceride levels have been observed in response to a diet extremely high in carbohydrate (34). Although our diet was not high in carbohydrate (42.6% of Kcal), we observed a significant triglyceride enrichment of VLDL coupled with a significant reduction in HDL-cholesterol levels during skim milk supplementation. However, these fluctuations never departed from the range of normalcy. Moderate consumption of alcohol increases HDL levels (35). From the subjects' food records alcohol consumption, equivalent to 1 to 15 oz. (mean 6 oz) of pure ethanol/wk, was recorded by all subjects. Since our experimental protocol did not permit the consumption of alcoholic beverages, the decline in HDL during the course of the study could simply reflect the removal of alcohol from the diet. High density lipoprotein-cholesterol appears to be the primary precursor for biliary cholesterol in man (36). Mitchell, Fyfe, and Smith (10) found an increase in fecal neutral sterol excretion during skim milk consumption in the elderly. It is conceivable that skim milk consumption may lower HDL-cholesterol by diverting it to the bile.

The plasma cholesterol:HDL-cholesterol ratio increased during skim milk ingestion; a change in this direction is not desirable considering the antiatherogenic property ascribed to HDL (37). However, during the skim milk period, the mean ratio, 3.6, was still well within the normal range (38). Perhaps more noteworthy was the lower mean ratio, 2.9, observed during the high calcium control period.

An increase in LDL-cholesterol occurred with skim milk consumption. In addition, total LDL lipid increased significantly, and LDL became enriched with surface components, notably free cholesterol. These trends are in a direction similar to the more drastic changes seen in LDL with parenchymal liver disease. The LDL become laden with free cholesterol, almost to the exclusion of esterified cholesterol (39). Simultaneously, HDL concentrations are reduced (39). The mechanisms underlying these changes are unclear, although a relative deficiency of lecithin:cholesterol acyltransferase, the plasma cholesterol esterifying

enzyme, precipitated by liver malfunction, is thought to be partially responsible (39). It should be emphasized that the LDL changes observed in the present study were by no means pathological. Nevertheless, it is tempting to speculate that this shift in LDL composition reflects subtle alterations in hepatic metabolism induced by the influx of the nutrient load imposed by consumption of two quarts of skim milk.

To conclude, we found that the ingestion of two quarts of fresh skim milk exerted a slight hypercholesterolemic effect under the diet conditions that mimicked the typical American diet pattern. However, the results suggest that healthy young men were able to maintain normal plasma cholesterol concentrations and desirable proportions of HDL-cholesterol in spite of the modest load of dietary cholesterol, fat and animal protein consumed in the experimental diets of this study.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the direction and consultation of Dr. Ralph Ellefson, Section of Clinical Chemistry, Department of Laboratory Medicine, Rochester, MN, who developed the ultracentrifugation technique to separate lipoproteins used in this study and the able technical assistance of Ms Karen Aschenbach and Elizabeth Johnson.

REFERENCES

1. FRIEND, B. and MARSTON, R. National Food Situation. USDA Economic Research Service #154. Washington, D.C., 1975.

2. CAMPBELL, R.G., HASKIM, S.A. and VAN ITALLIE, T.B. Effect of dairy fat and dairy fat fractions on serum lipids and cholesterol metabolism in man. In: Dairy Lipids and Lipid Metabolism. M.J. Brink and D. Kritchevsky (eds). AVI Publishing Co., Inc., Westprot, Conn., 1968, pp. 196-208.

3. Select Committee on Nutrition and Human Needs. U.S. Senate. Dietary Goals for the U.S. 2nd edition. U.S. Government Printing Office, Washington, D.C., 1977, pp. 1-4.

4. MANN, G.V. and SPOERRY, A. Studies of a surfactant and cholesteremia in the Maasai. Am. J. Clin. Nutr. 27: 464-469, 1974.

5. Mann, G.V. A factor in yogurt which lowers cholesteremia in man. Atherosclerosis 26: 335-340, 1977.

6. HOWARD, A.N. and MARKS, J. Hypocholesterolemic effect of milk. Lancet 2: 255-256, 1977.

7. HEPNER, G., FRIED, R., St. Jeor, S., FUSETTI, L. and MORIN, R. Hypocholesterolemic effect of yogurt and milk. Am. J. Clin. Nutr. 32: 19-24, 1979.

8. HOWARD, A.N. and MARKS, J. Effect of milk products on serum-cholesterol. Lancet 2: 957, 1979.

9. ROSSOUW, J.E., BURGER, E-M., VAN DER VYVER, P., and FERREIRA, J.J. The effect of skim milk, yoghurt, and full cream milk on human serum lipids. Am. J. Clin. Nutr. 34: 351-356, 1981.

10. MITCHELL, W.D., FYFE, T. and SMITH, D.A. The effect of oral calcium on cholesterol metabolism. J. Atheroscler. Res. 8: 913-922, 1968.

11. YACOWITZ, H., FLEISCHMAN, A.I. and BIERENBAUM, M.L. Effects of oral calcium upon serum lipids in man. Brit. Med. J. 1: 1352-1354, 1965.

12. ALBANESE, A.A., EDELSON, H., WOODHULL, M.L., LORENZE, E.J., WEIN, E.H. and ORTO, L.A. Effect of a calcium supplement on serum cholesterol, calcium, phosphorus and bone density of "normal, healthy" elderly females. Nutr. Rep. Int. 8: 119-130, 1973.

13. CARLSON, L.A., OLSSON, A.G., ORO, L. and ROSSNER, S. Effects of oral calcium upon the serum cholesterol and triglycerides in patients with hyperlipidemia. Atherosclerosis 14: 391-400, 1971.

14. BIERENBAUM, M.L., FLEISCHMANN, A.I. and RAICHELSON, R.I. Long term human studies on the lipid effects of oral calcium. Lipids 7: 202-206, 1972.

15. BHATTACHARYYA, A.K., THERA, C., ANDERSON, J.T., GRANDE, F. and KEYS, A. Dietary calcium and fat. Effect on serum lipids and fecal excretion of cholesterol and its degradation products in man. Am. J. Clin. Nutr. 22: 1161-1174, 1969.

16. National Academy of Sciences. Recommended Dietary Allowances. 8th edition. U.S. Government Printing Office, Washington, D.C., 1974.

17. MIETTINEN, T.A., AHRENS, JR., E.H. and GRUNDY, S.M. Quantitative isolation and gas-liquid chromatographic analysis of total dietary and fecal neutral steroids. J. Lipid Res. 6: 411-434, 1965.

18. SCALLER, D. Enzyme modified NDF method. In: The Nutritional Significance of Dietary Fiber, K.K. Kimura (ed). Bureau of Food, FDA, Washington, D.C., 1977.

19. GOERING, H.K. and VAN SOEST, P.J. Forage Fiber Analysis. Agriculture Handbook #379. U.S. Government Printing Office, Washington, D.C., 1970.

20. Perkin-Elmer, Corp. Analytical Methods for Atomic Absorption Spectrophotometry, Norwalk, Conn., 1976.

21. FISKE, C.H. and SUBBAROW, Y. The colorimetric determination of phosphorus. J. Biol. Chem. 66: 375-400, 1925.

22. ADAMS, C.F. Nutritive Value of American Foods. Agriculture Handbook #456. U.S. Government Printing Office, Washington, D.C., 1975.

23. FEELEY, R.M., CRINER, P.E. and WATT, B.W. Cholesterol content of foods. J. Am. Dietet. A. 61: 134-146, 1972.

24. CARPENTER, K.J., GOTSIS, A. and HEGSTED, D.J. Estimation of total cholesterol in serum by a micromethod. Clin. Chem. 3:233-238, 1957.

25. BLIGH, E.G. and DYER, W.J. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37: 911-917, 1959.

26. KRITCHEVSKY, D., DAVIDSON, L.M., KIM, H.K. and MALHOTRA, S. Quantification of serum lipids by a simple TLC charring method. Clin. Chim. Acta 46: 63-68, 1973.

27. KATES, M. Techniques of Lipidology. Elsevier Publishing Co., Inc., New York, 1972.

28. BARTLETT, G.R. Phosphorus assay in column chromatography. J. Biol. Chem. 234: 466-468, 1959.

29. FLETCHER, M.J. A colorimetric method for estimating serum triglycerides. Clin. Chim. Acta 22: 393-397, 1968.

30. FOSTER, L.B. and DUNN, R.T. Stable reagents for determination of serum triglycerides by a colorimetric Hantzch condensation method. Clin. Chem. 19: 338-340, 1973.

31. DRAPER, N.R. and SMITH, H. Applied Regression Analysis. John Wiley and Sons, Inc., New York, 1966, pp. 243-262.

32. HUSSI, E., MIETTINEN, T.A., OLLUS, A., KOSTIAINEN, E., EHNHOLM, C., HAGLUND, B., HUTTUNEN, J.K. and MANNINEN, V. Lack of serum cholesterol-lowering effect of skimmed milk and buttermilk under controlled conditions. Atherosclerosis 39: 267-272, 1981.

33. LEVY, R.I. High density lipoproteins, 1978 - an overview. Lipids 13: 911-913, 1978.

34. BLUM, C.B., LEVY, R.I., EISENBERG, S., HALL, M., GOEBEL, R.H. and BERMAN, M. HDL metabolism in man. J. Clin. Invest. 60: 795-807, 1977.

35. HULLEY, S.B., COHEN, R. and WIDDOWSON, G. Plasma high density lipoprotein cholesterol level. J. Amer. Med. Asso. 238: 2269-2271, 1977.

36. SCHWARTZ, C.C., HALLORNA, L.G., VLAHEVIC, Z.R., GREGORY, D.H., and SWELL, L. Preferential utilization of free cholesterol from HDL for biliary cholesterol secretion in man. Science 200: 62-64, 1978.

37. MILLER, N.E. The evidence for the antiatherogenicity of high density lipoprotein in man. Lipids 13: 914-919, 1978.

38. GORDON, T., CASTELLI, W.P., HJORTLAND, M.C., KANNEL, W.B. and DAWBER, T.R. High density lipoproteins as a protective factor against coronary heart disease. Am. J. Med. 62: 707-714, 1977.

39. MCINTYRE, N., CALANDRA, S. and PEARSON, A.J.G. Lipid and lipoprotein abnormalities in liver disease: the possible role of LCAT. Scand. J. Clin. Lab. Invest. Suppl. 137: 115-120, 1974.

NUTRITION RESEARCH, Vol. 1, pp. 443-448, 1981
0271-5317/81/050443-06$02.00/0 Printed in the USA.
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ABNORMAL DARK ADAPTATION IN ADULT PATIENTS WITH PROTEIN-ENERGY MALNUTRITION: CORRECTION BY PROTEIN-ENERGY REPLETION

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ABSTRACT

Markedly abnormal dark adaptation was observed in three patients with treated pancreatic insufficiency and one patient with histologically inactive alcoholic cirrhosis despite normal serum vitamin A (>40 µg/dl) and zinc levels (>70 µg/dl). Intrinsic retinal disease was excluded by extensive ophthalmological examinations in each case. Abnormal dark adaptation was associated with protein-energy malnutrition in all patients. In two patients, dietary protein repletion without supplemental vitamin A or zinc led to a rise in serum albumin and serum vitamin A transport proteins, and correction of the dark adaptation abnormality. No change was observed in total serum vitamin A and serum zinc levels after protein repletion for six weeks. These observations may indicate impaired peripheral utilization of retinol in chronically ill patients with protein-energy malnutrition.

KEY WORDS: Dark adaptation, protein-energy malnutrition, pancreatic insufficiency, cirrhosis, retinol, zinc.

INTRODUCTION

Abnormal dark adaptation occurs frequently in patients with gastrointestinal and hepatic diseases, and is generally correctable by vitamin A supplements (1, 2). However, as early as 1938, Lasch observed abnormal dark adaptation in some patients with cirrhosis despite normal serum vitamin A values (3). This observation raised the possibility that vitamin A was not the only factor necessary for normal night vision. In 1978, oral zinc supplements were shown to correct abnormal dark adaptation in six patients with cirrhosis and zinc deficiency, suggesting that zinc may also be important in the integrity of night vision (4, 5).

We have recently encountered three patients with pancreatic insufficiency and one patient with alcoholic cirrhosis who had persistently abnormal dark adaptation despite normal serum vitamin A and zinc levels. All patients were chronically ill and had evidence for protein-energy malnutrition. Since abnormalities of vitamin A transport system have been described in children with protein-energy malnutrition (PEM), we conjectured that abnormal dark adaptation in our four patients might be related to PEM due to impaired vitamin A transport (6). To investigate this possibility we studied the serum vitamin A transport system in all four patients after excluding significant intrinsic retinal disease by extensive ophthalmologic examinations. Further, the effect of protein repletion on the final dark adapted threshold and vitamin A transport system was examined in two of the four patients.

PATIENTS AND METHODS

Three patients with pancreatic insufficiency secondary to chronic alcoholic pancreatitis and one patient with alcoholic cirrhosis were studied. All patients were male with a mean age of 53 years (range 40-57). The diagnosis of pancreatic insufficiency was based on the presence of the following four criteria: a) presence of steatorrhea, b) abnormal secretin test, c) normal d-xylose absorption, and d) favorable clinical response to oral pancreatic enzyme therapy. A favorable clinical response was defined as at least a ten pound weight gain coupled with 50% decrease in fecal fat excretion on oral pancreatic enzyme therapy. Patients with pancreatic disease had been taking ten tablets of Pancreatin (Eli Lilly & Co., Indianapolis, IN) with each meal three times daily for one to ten years, and 15 to 25 units NPH insulin for three to four years. The diagnosis of alcoholic cirrhosis was based on the characteristic histologic features of the liver biopsy.

Moderate steatorrhea was present in all four patients as indicated by a mean (± SEM) fecal fat excretion of 23 ± 12 g/day. None of the patients had ingested ethanol for at least six months prior to the clinical investigation. During this period, each patient was on a diet which was estimated by a dietitian to be adequate in vitamin A content (recommended daily allowance: 1650 ug). None had received multi-vitamin tablets or vitamin A supplements in the four months prior to study.

Dark adaptation was measured in each patient by a Goldman-Weekers adaptometer (Haag-Streit, Bern, Switzerland) using a previously described method (2). Total serum vitamin A was measured by trifluoroacetic acid method of Neeld and Pearsen (7). Retinol and retinyl esters were extracted from plasma, separated from each other on a deactivated alumina column and measured fluorometrically (8). Prealbumin and retinol binding protein were determined by radial gel diffusion using commercially available kits (Behring Calbiochem Labs, Summerville, NJ). Zinc concentrations were measured on a Perkin-Elmer atomic absorption spectrophotometer (model 303, Perkin-Elmer Corporation, Norwalk, CN) (9). Blood for zinc determinations was collected with zinc-free plastic syringes and stainless steel needles. All four patients underwent an eye examination which included: i) fundus examination, ii) visual fields, iii) Snellen acuity, and iv) intraocular pressure determinations. Additionally, fluorescein angiography was performed on three patients with pancreatic insufficiency.

Two of the four patients (Nos. 2 and 3) were placed on high protein (150 g/day), high calorie (3,500 cal/day) diet for six weeks. The effect of protein-calorie repletion without vitamin A and zinc supplementation on the dark adaptation abnormality and the transport of serum retinol was studied.

RESULTS

A marked abnormality of final dark adaptation threshold was initially observed in all four patients (Table I). However, total serum vitamin A and zinc levels were well within normal range. Furthermore, serum retinol binding protein and prealbumin levels were abnormal only in patient No. 3, and these were borderline low values. Serum retinyl esters were mildly elevated ranging from 7.0 to 13.2 µg/dl (normal range 0.0 to 7.1 µg/dl).

All patients had normal Snellen visual acuity, intraocular pressure and visual field examinations. Fundus examination was also normal in all. Two to three microaneurysms were seen on fluorescein angiography in two patients (Nos. 1 and 2). No equatorial punctate lesions were seen in patients with pancreatic disease.

All patients had evidence of protein-energy malnutrition. Three patients (Nos. 2-4) measured less than 70% of ideal body weight (10,11). Serum albumin and prealbumin levels were borderline low normal, or low in all patients (Table I).

TABLE I

Clinical and Biochemical Features of Four Patients with Protein-Energy Malnutrition and Abnormal Dark Adaptation

SND	Diagnosis	DA[a] Final Threshold ($\log cd/m^2$)	Total Serum vA[b] (μg/dl)	Serum Zinc (μg/dl)	RE[c] (μg/dl)	RBP[d] (μg/ml)	PA[e] (μg/ml)	Serum Albumin (g/dl)
1.	PI[f]	-3.8	52	108	8.5	40.6	180	3.7
2.	PI	-3.6	52	108	9.4	34.5	180	2.5
3.	PI	-3.7	51	106	13.3	28.5	135	3.7
4.	AC[g]	-4.3	50	90	7.0	41.0	101	2.6
Normal Range[h]		-4.4 to -5.2 (n=28)	27-83 (n=124)	74-122 (n=64)	0-7.1 (n=26)	29-81 (n-167)	166-406 (n-165)	3.8-5.0 (n=100)

a = Dark Adapted (log candela per square meter)

b = Vitamin A

c = Serum Vitamin A as Retinyl Esters

d = Serum Retinol Binding Protein

e = Serum Prealbumin

f = Pancreatic Insufficiency

g = Alcoholic Cirrhosis

h = Normal Range: range of values based on mean ±2 SD in normal control subjects.

Final dark adapted thresholds returned to normal in two patients after receiving a high protein diet for six weeks (Fig. 1). This observation was associated with normalization of serum albumin and a mild elevation in retinol binding protein and prealbumin levels. Total serum vitamin A ester levels decreased from 9.4 to 13.3 μg/dl to 3.5 and 4.1 in both patients, respectively. Additionally, protein repletion was accompanied by a 2-3 kg weight gain in both patients.

DISCUSSION

Marked abnormality of final dark adapted thresholds is frequently observed in patients with vitamin A deficiency (2). More recently, zinc deficiency has been shown to be associated with impaired dark adaptation (4,5). Four patients reported in this communication revealed severely impaired dark adaptation despite normal levels of vitamin A and zinc in the serum. Intrigued by this observation, we studied each patient ophthalmologically to evaluate the retina, both structurally and functionally. Microaneurysms were observed on fluorescein angiography in two patients (Nos. 1 and 2). However, these minimal vascular changes of diabetic retinopathy cannot explain the markedly elevated final dark adapted thresholds seen in our patients, since diabetic patients without significant retinopathy have been demonstrated to have normal dark adaptation tests (12). Furthermore, normalization of the dark adaptation abnormality in two patients on protein repletion argues against the possibility of abnormal dark adaptation

being due to diabetic retinopathy. Interestingly, none of our three patients with chronic pancreatitis revealed non-diabetic retinal abnormalities recently described by Toskes et al in a group of patients with chronic pancreatitis (13).

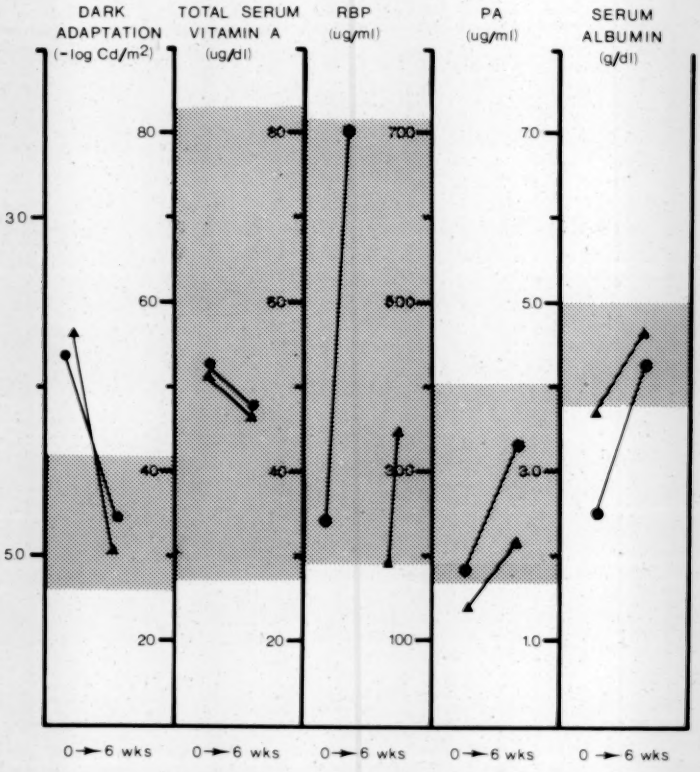

FIG. 1

Effect of dietary protein calorie repletion of six weeks duration on dark adaptation, total serum vitamin A, retinol binding protein, prealbumin, and albumin in two patients with protein-energy malnutrition due to pancreatic insufficiency. Patients Nos. 2 and 3 are represented by (●) and (▲), respectively.

Baseline serum vitamin A as retinyl esters were mildly elevated in three of four patients, which decreased to normal in two patients on protein repletion. It has been postulated by Smith et al that vitamin A toxicity occurs when the capacity of retinol binding protein to transport vitamin A is exceeded and excessive amounts of vitamin A are presented to cell membrane in the unbound form (14). Retinol not bound to protein has been shown to lead to an increased synthesis and release of hydrolases from lysosomes (15,16). The hydrolases are considered to be important mediators of the deleterious effect of vitamin A on cartilage and bone (17). Thus it is conceivable that the administration of vitamin A in high doses to patients with protein calorie malnutrition may predispose them to vitamin A toxicity.

Serum retinol binding protein was well within the normal range in three pa-

tients and only borderline low in one (patient No. 3). Since serum vitamin A levels were normal in all four patients, this observation would suggest normal transport of vitamin A to the peripheral sites. However, abnormal dark adaptation in this group of patients might be explained by impaired peripheral utilization of vitamin A. The complex process of peripheral utilization of vitamin A is dependent on the cellular uptake and intracellular transport of vitamin A (18,19). For the cellular uptake of vitamin A, the presence of specific protein receptors for retinol binding protein has been conclusively demonstrated on the choroidal surface of bovine and human pigment epithelial cells (20,21). Furthermore, intracellular retinol binding protein which is distinct from circulating retinol binding protein has been recently described which may be essential for the interaction of retinol with the cell nucleus (18).

We postulate that impaired dark adaptation in our four patients may be related to specific defect at one or more of the essential steps in the peripheral metabolism of vitamin A. Although the deficiency of other micronutrients such as riboflavin, ascorbic acid and panthothenic acid can lead to abnormal dark adaptation, the likelihood of such an occurrence in all four patients is remote. None of our patients demonstrated overt clinical manifestation of deficiency of these vitamins. Protein repletion in our patients was not accompanied by changes in serum vitamin A or zinc levels although serum retinol binding protein levels showed a moderate elevation. The effect of protein depletion and repletion on the specific cellular receptors for retinol binding protein and intracellular retinol binding protein need further investigation. Additionally, studies are needed to precisely identify single or multiple steps in the complex process of human vision which may individually or collectively be affected by malnutrition.

ACKNOWLEDGMENTS

The authors gratefully thank R. A. Jacobs, Ph.D. of the Human Nutrition Laboratory, Grand Forks, ND for performing serum retinol binding protein and prealbumin assays. We also wish to thank Y. Ferguson, A. Brzeczko and M. Garrett-Laster for technical help, and H. Spencer, P. Weinel, T. Baker, M. A. Pirog, and A. Caliger for assistance in the preparation of this manuscript.

This research was supported by the Veterans Administration.

REFERENCES

1. PATEK, A.J. and HAIG, C. The occurrence of abnormal dark adaptation and its relation to vitamin A metabolism in patients with cirrhosis of the liver. J. Clin. Invest. 18:609-616, 1939.

2. RUSSELL, R.M., SMITH, V.C., MULTACK, R., KRILL, A.E. and ROSENBERG, I.H. Dark adaptation testing for diagnosis of subclinical vitamin A deficiency and evaluation of therapy. Lancet 2:1161-1164, 1973.

3. LASH, F. Uber den Vitamin A-Spiegel im Blute bei Leberkrankheiten. Klin. Wchnshr. 17:1107-1108, 1939.

4. RUSSELL, R.M., MORRISON, S.A., SMITH, F.R., OAKS, E.V. and CARNEY, E.A. Vitamin A reversal of abnormal dark adaptation in cirrhosis. Study of effects on the plasma retinol transport system. Ann. Intern. Med. 88:611-626, 1978.

5. MORRISON, S.A., RUSSELL, R.M., CARNEY, E.A. and OAKS, E.V. Zinc deficiency: A cause of abnormal dark adaptation in cirrhotics. Am. J. Clin. Nutr. 31(2): 276-281, 1978.

6. SMITH, R.R., GOODMAN, D.S., ZAKLAMA, M.S., GABR, M.K., MARAGHY, S.E. and PATWARDHAM, V.N. Serum vitamin A, retinol-binding protein-calorie malnutrition. I. A functional defect in hepatic retinol release. Am. J. Clin. Nutr. 26:973-981, 1973.

7. NEELD, J.B. JR. and PEARSEN, W.N. Macro and micromethods for the determination of serum vitamin A using trifluoroacetic acid. J. Nutr. 79:454-462, 1963.

8. MALLIA, A.K., SMITH, J.E. and GOODMAN, I.S. Metabolism of retinol binding protein and vitamin A during hypervitaminosis A in the rat. J. Lipid Res. 16:180-188, 1975.

9. PEKAREK, R., BEISEL, E.O., BARTELLONI, P.J. and BOSTIAN, K.A. Determination of serum zinc concentrations in normal adult subjects by atomic absorption spectrophotometry. Am. J. Clin. Pathol. 57:506-510, 1972.

10. JELLIFFE, D.B. The assessment of the nutritional status of the community. Geneva: World Health Organization, 1966.

11. Assessment of Protein Nutritional Status: A Committee Report. Am. J. Clin. Nutr. 23:807-819, 1970.

12. ASPINALL, P.A. Rod-cone interaction: Some indirect evidence. Acta. Ophthalmol. 55:296-302, 1977.

13. TOSKES, P.P., DAWSON, W., CURINGTON, C., LEVY, N.S. and FITZGERALD, C. Non-diabetic retinal abnormalities in chronic pancreatitis. N. Engl. J. Med. 300:942-946, 1979.

14. SMITH, F.R. and GOODMAN, D.W. Vitamin A transport in human vitamin A toxicity. N Engl. J. Med. 294:805-808, 1976.

15. DINGLE, J.T. Vacuoles, vesicles and lysosomes. Brit. Med. Bull. 24:141-145, 1968.

16. ROELS, O.A., ANDERSON, O.R., LUI, N.C., SHAH, D.O. and TROUT, M.E. Vitamin A and membranes. Am. J. Clin. Nutr. 22:1020-1032, 1969.

17. FELL, H.B. and DINGLE, J.T. Studies on the mode of action of excess vitamin A. VI lysosomal protease and the degradation of cartilage matrix. Biochem. J. 87:403-408, 1963.

18. CHYTIL, R. and ONG, D.E. Cellular retinol and retinoic acid binding protein in vitamin A action. Fed. Proc. 38:2510, 1979.

19. HELLER, J. Interaction of plasma retinol binding protein with its receptors. Specific binding of bovine and human retinol binding protein to pigment epithelial cells from bovine eyes. J. Biol. Chem. 250:3613, 1975.

20. CHEN, CHI-CHING and HELLER, J. Uptake of retinol and retinoic acid from serum retinol binding protein by retinal pigment epithelial cells. J. Biol. Chem. 252:5216-5221, 1977.

21. BERGSMA, D.R., WIGGERT, B.N., FUNAHASHI, M., KUWABARA, T. and CHADER, G.J. Vitamin A receptors in normal and dystrophic human retina. Nature 265:66-67, 1977.

NUTRITION RESEARCH, Vol. 1, pp. 449-459, 1981
0271-5317/81/050449-11$02.00/0 Printed in the USA.
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HUMAN MILK BANKING I. EFFECTS OF CONTAINER UPON IMMUNOLOGIC FACTORS IN MATURE MILK[1,2]

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ABSTRACT

The effects of the type of storage container and the duration of storage upon the concentrations of lactoferrin, lysozyme, secretory IgA (SIgA), total IgA, and SIgA antibodies to E.coli somatic antigens and upon the numbers and functions of macrophages, neutrophils and lymphocytes in mature human milk were examined. Milk samples collected during the fourth week of lactation from women aged 20-35 years were kept in pyrex and polypropylene or polyethylene containers at 4°C for 4 and 24 hours. The concentrations of lactoferrin and lysozyme fell by 30% and 40%, respectively, after storage for 24 hours in pyrex and polypropylene containers. The concentration of SIgA antibodies to E.coli O antigens did not change with time in samples stored in pyrex or polypropylene containers but fell by 60% in samples stored in polyethylene. The numbers of macrophages and neutrophils were similar in all containers after 4 hours of storage but increased after 24 hours, particularly after storage in pyrex containers. A similar pattern was found in respect to the number of lymphocytes. The uptake of ^{3}H-thymidine by phytohemagglutinin stimulated milk lymphocytes decreased significantly after 24 hours of storage. These findings indicate that the storage of mature milk at 4°C for comparatively brief periods induces significant changes in the concentrations and/or functions of key immunological components.

KEY WORDS: Human milk, lysozyme, lactoferrin, secretory IgA, leukocytes.

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[1] The publication of this work is supported by a contract from the National Institutes of Child Health and Human Development DHEW #1-HD-8-2828 and by the United States Department of Agriculture, Science, and Education Administration, Children's Nutrition Center, Department of Pediatrics, Baylor College of Medicine and Texas Children's Hospital.

[2] Presented in part at the International Symposium on Breast Feeding, Tel Aviv, Israel, February 25-28.

INTRODUCTION

In the latter half of the 20th century, renewed interest has developed in the superiority of human milk in infant nutrition. The conclusion that human milk is superior is based upon epidemiologic studies in industrial as well as developing countries (1-5). Although no one study is compelling, these investigations indicate that breast-fed infants are less prone to gastrointestinal and respiratory infections, and that mortality rates among breast-fed infants are greatly reduced.

Major differences have been found between mature human milk and cow's milk, the principal substitute for human milk in infant nutrition (6-11). Because of the superiority of human milk and its unique composition, banks have been established to provide milk to infants who would not otherwise receive it. There is comparatively little information, however, concerning the effects of different donor populations and storage conditions on the quality or quantity of the components of human milk (12-15).

In 1978 we began a multidisciplinary study with an objective to provide information that would aid in establishing standards for banking human milk. In this initial report, the effects of storage container upon certain immunologic components of mature human milk will be presented.

METHODS

Research Assurance: The research was approved by the Institutional Review Boards of both medical schools and each individual freely gave consent before participating in the research.

Selection of Subjects: The donors were women 20-35 years of age who had one or two previous pregnancies and whose infants were full-term. These mothers were from a middle to upper socioeconomic population that utilized private medical services. The last infant was exclusively breast-fed. The mothers were in good health. Their milk production was judged to be adequate by the growth of their nursing infants. No attempt was made to select individuals with an over-abundant supply of milk. The diets of the donors contained a wide assortment of meats, fruits, and vegetables. Smokers were excluded. The mothers denied the use of drugs during the week before donation.

Study Design and Collection Procedures: The study was conducted in two phases. In the first phase, rigid pyrex glass cylinders (Egnell, Inc., Cary, IL) were compared with flexible polyethylene bags (Gerber Products, Freemont, MI). In the second phase, pyrex glass containers were compared with rigid polypropylene containers (Egnell, Inc.). The number of subjects in each comparison is stipulated in the Results (See Figs.). The pyrex and polypropylene containers were washed in a detergent, rinsed 8-10 times with deionized water, and then sterilized by autoclaving, whereas the polyethylene containers were not treated. Milk samples were obtained during the fourth week of lactation between 8:30 a.m. and 12:00 noon each day by use of an electrical pump (Egnell, Inc.) that provides a reliably low, intermittent pressure for stimulating the flow of milk. Specimens were collected 1-3 h following the last nursing. The breast was

emptied of milk with each collection. The collections were usually completed within 10 minutes. Each donor contributed two samples from the same breast with a 2-day interval between collections. One sample was stored in a pyrex glass cylinder and the other in either a polyethylene bag or polypropylene container. The type of container used for the first collection was assigned randomly. Plastic or pyrex glass nipple shields were chosen to correspond with the type of collection vessels used. All samples were stored immediately at 4°C and transported in an ice chest with minimal agitation. Aliquots were obtained from each sample at 4 and 24 h to test for the effect of duration of storage. After stirring with a glass rod, a portion of each aliquot was removed for cell enumeration. The remainder of each aliquot was diluted with two volumes of Hank's balanced salt solution and centrifuged at 300XG for ten minutes. Fat layers were decanted and the remaining fluids were frozen at -20°C until assayed for soluble immunologic components. Prior studies showed that freezing samples up to 3 months did not affect the results of these assays. The pellet was used immediately in cell function studies described below.

Measurement of Immunologic Components: Lysozyme and lactoferrin were quantified by electroimmunodiffusion (16). Monospecific antisera to these proteins were obtained commercially (Calbiochem-Behring Corporation, La Jolla, CA). A human urinary lysozyme preparation (Kallestad Laboratories, Chaska, MN) and a lactoferrin preparation isolated from human colostrum were used as standards (17). The relationships between the height of the "rockets" and protein concentrations were linear for lysozyme concentrations between 8 and 32 µg/ml. The average coefficient of variation for 18 duplicate determinations was 8.0% for lysozyme and 11.7% for lactoferrin.

To measure SIgA in the presence of other molecular forms of IgA, a previously described fluorescent immunosorbent technique was utilized (18,19). The total IgA content of the sample is measured using an antihuman α chain fluorescent detector and standard curves for a purified SIgA and a standard human serum that contained known quantities of serum IgA. SIgA measurements utilized fluorescenated anti-secretory component antiserum as the detector and purified SIgA as the standard. The concentration of IgA and SIgA was linearly related to the relative fluorescence between 48 and 480 ng and 49 and 488 ng per assay tube, respectively. The relationship between total and SIgA was expressed by the equation:

$$IgA_T = K_2 \cdot RF_T + [SIgA] - \frac{K_1}{K_2} [SIgA]$$

where IgA_T is the total IgA, SIgA is secretory IgA, RF_T is the relative fluorescence of total IgA (e.g., anti-α chain conjugate), and K_1 and K_2 are the slopes of the standard curves for SIgA and serum IgA in the total IgA assay. The average coefficient of variation for 26 determinations was 19.5% for total IgA and 19.0% for SIgA.

Secretory IgA antibodies to E.coli antigens were measured utilizing somatic (O) antigens from common strains of E.coli (20). A micro-modification of an enzyme-linked immunosorbent assay was adapted to measure SIgA antibodies by utilizing an anti-human

secretory component antiserum globulin conjugated to alkaline phosphatase (21). The results were expressed as the reciprocal of the titer at half-maximum enzyme activity. Whenever possible, all samples from a single individual were assayed simultaneously. The average coefficient of variation for 20 determinations was 21% when the duplicate determinations were on the same plate and 57% when the sample was assayed twenty times on different plates.

Viable cells were enumerated microscopically using a hemocytometer (American Optical Corporation, Buffalo, NY) and the exclusion of trypan blue (Matheson, Coleman, and Bell, Norwood, OH). For these counts, aliquots of milk were obtained with polypropylene pipettes (Oxford No. 819-S, Oxford Laboratories, Foster City, CA). The proportions of macrophages, neutrophils, and lymphocytes were confirmed by microscopic examination of Giemsa stained preparations. Because of the difficulty in distinguishing fat-laden macrophages and neutrophils, the counts of these cells were combined.

Cells were prepared for functional studies in the following manner. Pellets formed after centrifugation of the diluted aliquots were resuspended and washed with a mixture of RPMI-1640 (Microbiological Associates, Los Angeles, CA), 12% fetal calf serum (Flow Laboratories, McLean, VA), Hepes buffer (Pacific Biological Co., Downer Grove, IL), penicillin G and streptomycin. Cell counts were performed as previously described.

The ^{3}H-thymidine incorporation by stimulated lymphocytes was measured by a microculture assay. Unstimulated preparations consisted of 50 µl of the cells (50,000 lymphocytes) and 150 µl of the previously described media mixture; stimulated preparations consisted of 50 µl of the cells, 125 µl of the media, and 25 µl of phytohemagglutinin-P (PHA-P)(3 µg)(Difco Lab, Detroit, MI). These preparations were incubated in flat-bottom microtiter wells for 72 h in 3-5% CO_2 at 37°C. One micocurie of ^{3}H-thymidine was added to each well 4 h before harvest on glass fiber filters (Whatman, Inc., Clifton, NJ). The uptake of the isotope was measured by scintillation spectrometry.

To measure phagocytosis of opsonized Candida albicans, cells were resuspended in the media mixture in a concentration of 2.5 x 10^5 macrophages and neutrophils/ml. The cell suspension (0.2 ml) was placed on a coverglass in petri dishes and incubated for 20 min. at 37°C. The coverglasses were rinsed to remove non-adherent cells. Immediately thereafter, each coverglass was covered with 0.8 ml of a suspension of heat-killed Candida albicans prepared by mixing 0.5 ml of 1 x 10^7 organisms, 1 ml of human AB serum and 3.5 ml of a 12% fetal calf serum in RPMI-1640. The preparations were incubated for 30 min. at 37°C, rinsed, fixed with 5% gluteraldehyde, and stained with Giemsa. The percent of macrophages and neutrophils with ingested organisms was determined microscopically.

Statistical Methods: A three way analysis of variance, a mixed model for random (individual) and fixed effects (time and container), was used to evaluate the results. The model used was:

$$Y_{ijk} = \mu + \alpha_i + \beta_j + \gamma_k + \beta\gamma_{jk} + \varepsilon_{ijk}$$

where for the i^{th} individual, the j^{th} container, and the k^{th}

storage time, Y_{ijk} is the measured concentration of the given immunologic factor, μ is the overall mean for the factor being measured, α_i is the effect due to individual variation, β_j is the effect due to container, γ_k is the effect due to time of storage, $\beta\gamma_{jk}$ is the effect due to interactions between the storage container and time, and ε_{ijk} are effects due to unexplained sources of error. This model was used to test the null hypothesis of "no effect" at the 0.05 level. The alternate hypothesis, that treatment levels differed by at least one SD, was considered in determining that eleven subjects would be required to achieve the power, $1-\beta$, of 0.9. In the presentation of results, the SD of Y_{ijk} was calculated when population variability was being described. Standard errors of the mean (SEM) were calculated when treatment effects were compared.

RESULTS

The volume of each milk collection was measured. No significant difference was found in the amounts of milk collected for each container study.

The concentrations of lysozyme and of lactoferrin in milk stored for 4 h in each type of container were similar. The concentrations of these proteins did not change after 24 h of storage in polyethylene bags. The 4 and 24 h data were, therefore combined (overall mean ± SD for lysozyme in milk stored in polyethylene bags for 4 and 24 h, 32 ± 22 µg/ml; overall mean ± SD for lactoferrin in milk stored in polyethylene bags for 4 and 24 h, 2.2 ± 2.1 mg/ml). The concentrations of these proteins decreased, however, after storage for 24 h in pyrex glass or polypropylene (Fig. 1).

The concentrations of total IgA and SIgA in milk were not altered by the type of container or length of storage. The mean ± SD for both total IgA and for SIgA in all containers was the same (1.3 ± 0.6 mg/ml). The majority of IgA in each sample was SIgA (mean, 96%). The proportion of SIgA in the milk did not change after storage regardless of the container. The amounts of SIgA antibodies to E.coli O antigens in milk stored in pyrex and polypropylene were similar at 4 h and did not change appreciably after storage for 24 h (reciprocal titer, overall mean ± SD, 20 ± 33). In contrast, the levels of these antibodies in polyethylene containers were greatly reduced (Fig. 2). Similar statistical differences were found with log transformation of the data.

The numbers of macrophages and neutrophils were similar in each type of container after 4 h, but the numbers of these cells increased in the fluid after 24 h (Fig. 3). These changes were particularly marked in specimens stored in pyrex glass containers. A similar pattern was found in respect to the numbers of lymphocytes (Fig. 3).

The phagocytic capability of cells in milk stored for 4 h was similar in pyrex glass and both types of plastic containers (mean ± SD, 87 ± 9% and 86 ± 2%, respectively). Most cells in these preparations had ingested 7 or more organisms. Few macrophages or neutrophils adhered to glass coverslips after 24 h incubation at 4°C. Consequently, quantitative studies of phagocytosis were

not possible under those conditions.

The incorporation of ^{3}H-thymidine into PHA-P stimulated lymphocytes was not affected per se by the type of container (mean ± SEM for all containers after 4 h incubation, 4079 ± 1489 cpm) but the degree of stimulation diminished significantly after storage for 24 h (mean ± SEM, 591 ± 336 cpm; $p < 0.05$).

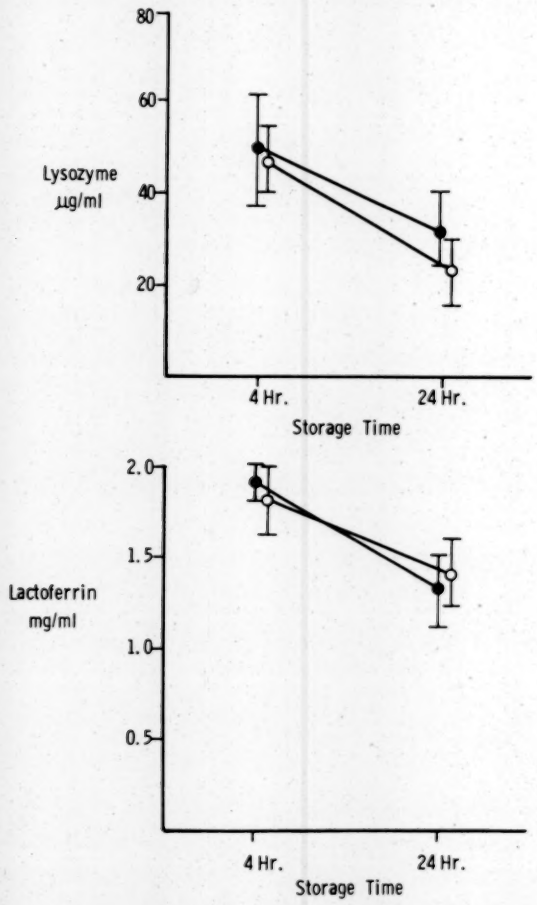

Figure 1. The concentrations of lysozyme and lactoferrin in human milk stored at 4°C (mean ± 1 SEM). Fifteen paired samples were used to compare lysozyme in pyrex glass (●) and polypropylene containers (o). Fourteen paired samples were used to compare lactoferrin in these containers. Significant differences due to time were found ($p < 0.01$).

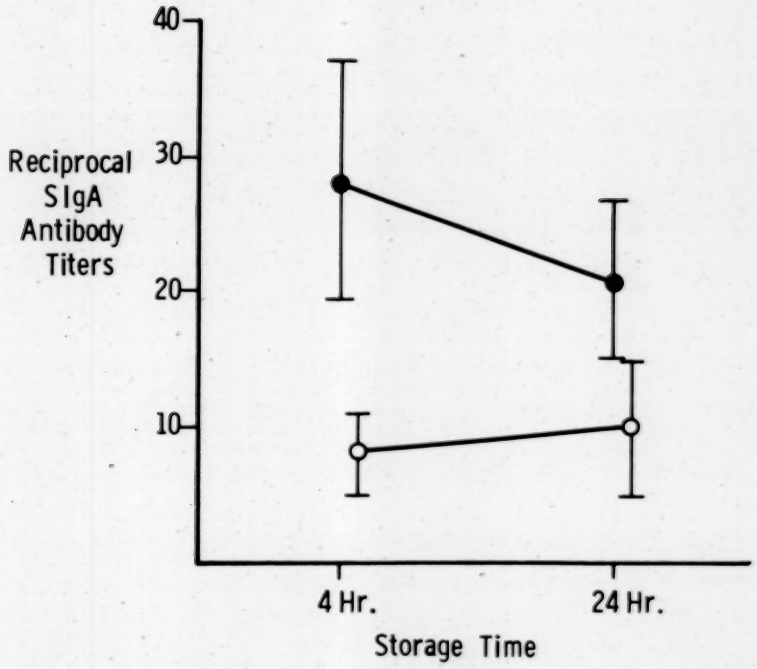

Figure 2. The levels of SIgA antibodies to E.coli O antigens after storage at 4°C (mean ± 1 SEM) in pyrex glass (●) and polyethylene (o) containers. Five paired samples were used in this study. Significant differences due to containers were found ($p < 0.05$).

Significant positive correlations ($p < 0.01$) were found between the concentrations of total IgA and the following parameters: the concentrations of SIgA ($r=0.84$), reciprocal titers of SIgA antibodies to E.coli O antigens ($r=0.43$), and the quantities of lactoferrin ($r=0.53$). Significant positive correlations ($p < 0.01$) were also found between the concentrations of lactoferrin and lysozyme ($r=0.56$) and between the numbers of macrophages-neutrophils and lymphocytes ($r=0.45$).

DISCUSSION

It has been reported that certain immunologic components of human milk are affected by comparatively high or low temperatures. Milk leukocytes are destroyed by freezing, holder pasteurization, or boiling (14). Holder pasteurization destroys much of the lactoferrin but does not affect the levels of IgA, lysozyme, or vitamin B12 or folic acid binding proteins (12). A detailed analysis of the effects of refrigeration and the type of container upon the immunologic factors in human milk has not been reported. In this study, the effects of pyrex glass, polyethylene, and polypropylene containers upon the storage of immunologic components of mature human milk at 4°C were examined. Stringent efforts were made to control demographic factors by choice of subjects. The

statistical procedures minimized variation due to individual differences. Infradian variations were lessened by collecting at the same time each day.

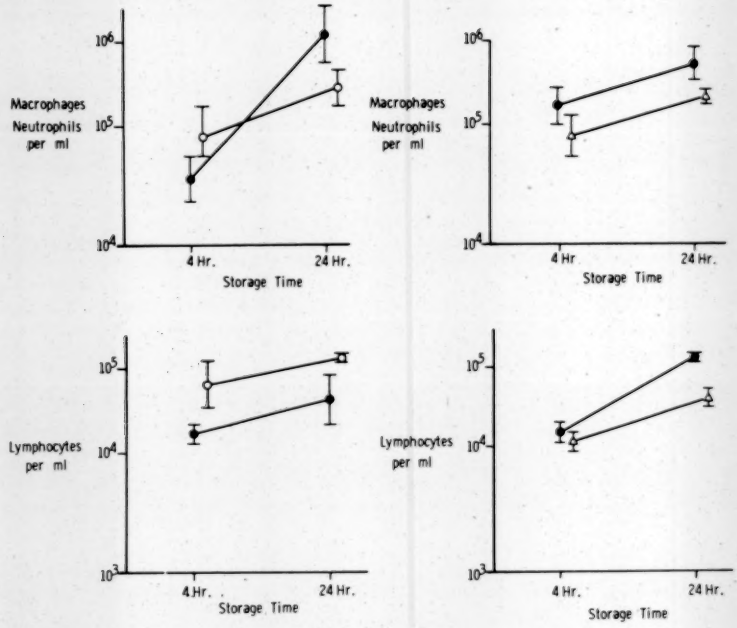

Figure 3. The concentrations of macrophages and neutrophils and lymphocytes in human milk stored at 4°C (mean ± 1 SEM). Seven paired samples were used to compare pyrex glass (●) and polyethylene containers (o). Eleven paired samples were used to compare pyrex (●) and polypropylene (Δ) containers. Significant differences due to time ($p < 0.05$) were found with each type of container. Significantly more macrophages, neutrophils, and lymphocytes were found in the fluid phase when milk was stored in pyrex glass as compared to polypropylene containers ($p < 0.05$).

Lysozyme and lactoferrin concentrations fell substantially after storage for 24 h in pyrex or polypropylene containers. It was not ascertained whether this was due to adherence of proteins to the vessel surface, proteolysis, or other mechanisms. In contrast, levels of IgA and SIgA were not altered. Secretory IgA antibodies to E.coli O antigens, however, were lower in polyethylene containers, presumably because of selective binding to the container surface.

The numbers of living macrophages, neutrophils, and lymphocytes in the fluid phase were similar after 4 h at 4°C regardless of the container. After 24 h, however, the numbers of macrophages,

neutrophils, and lymphocytes in the fluid phase increased significantly. Since no evidence was found for mitosis of the macrophages or neutrophils and since the proliferation of unstimulated lymphocytes was limited, these increments most likely were due to cells released from the container surface during the incubation. The reason for the loss of adherence of the cells is being investigated.

Certain functions of milk leukocytes were also affected by storage. The uptake of ^{3}H-thymidine by PHA-P stimulated milk lymphocytes decreased significantly after 24 h storage. Although the cause for this decrease was not ascertained, it appeared to be due to an inherent change in the cells or in available cell populations (9). Most cells in milk stored for 4 h at 4°C in each container phagocytized heat-killed Candida albicans. The cells recovered in the fluid after 24 h storage were unable to adhere to glass. Thus, it was not possible to assay the phagocytic capability of these cells after prolonged incubation in the cold.

The storage of mature human milk at 4°C for comparatively brief periods induces significant changes in the concentrations or function of key immunologic components. Although it is not possible to sample milk prior to contact with an artificial container, the alterations due to the type of container and the two storage periods studied indicate that stored milk cannot be equated with suckled breast milk. The biological significance of these changes in stored mature milk is undetermined. In particular, it is unknown whether the protection afforded to the infant will be reduced.

Since the increased number of cells recovered after prolonged incubation had significantly diminished function, we concluded that the shortest possible period of storage would be preferable in clinical and in vitro studies of these human milk preparations. Although none of the tested containers proved to be superior for the storage of all the measured immunologic factors, the profound loss of SIgA antibodies in polyethylene bags, the difficulty in handling the flexible bags, and the potential for breakage of the pyrex glass containers led us to prefer polypropylene containers for future studies in human milk banking.

ACKNOWLEDGEMENTS

The technical assistance of M. A. Armendariz, D. Nannen, and M. Douglas; the nursing assistance of E. Cherry, J. Jones, D. Mitchell, P. Perez, and J. Smith; the secretarial help from T. Creech and M. Boyd; and the editorial assistance of E. R. Klein were greatly appreciated. The E.coli somatic antigens were generously supplied by Lars A. Hanson of Goteborg, Sweden.

REFERENCES

1. Cunningham, A.S. Morbidity in breast-fed and artificially fed infants. J. Pediatr. 90: 726-729, 1977.

2. Cunningham, A.S. Morbidity in breast-fed and artificially fed infants II. J. Pediatr. 95: 685-689, 1979.

3. Grulee, C.G., Sanford, H.N., and Herron, P.H. Breast and

artificial feeding: Influence on morbidity and mortality of twenty-thousand infants. J. Am. Med. Assoc., 103: 735-739, 1934,

4. Larsen, S.A. and Homer, D.R. Relation of breast vs. bottle-feeding to hospitalization for gastroenteritis in a middle-class U.S. population. J. Pediatr., 92: 417-418, 1978.

5. Mata, L.J. and Urrutia, J.J. Intestinal colonization of breast-fed children in a rural area of low socioeconomic level. Ann. N.Y. Acad. Sci., 176: 93-109, 1971.

6. Goldman, A.S. and Goldblum, R.M. Anti-infective properties of human milk. Pediatr. Update, 2:359-367, 1980.

7. Goldman, A.S. and Smith, C.W. Host resistance factors in human milk. J. Pediatr., 82: 1082-1090, 1973.

8. Nichols, B.L. and Nichols, V.N. Lactation. Adv. Pediatr., 26: 137-161, 1979.

9. Ogra, S.S. and Ogra, P.L. Immunologic aspects of human colostrum and milk II. Characteristics of lymphocyte reactivity and distribution of E-rosette forming cells at different times after the onset of lactation. J. Pediatr., 92: 550-555, 1978.

10. Peiterson, B., Bohn, L., and Andersen, H. Quantitative determination of immunoglobulins, lysozyme and certain electrolytes in breast milk during the entire period of lactation, during a 24-hour period and in milk from the individual mammary gland. Acta. Paediatr. Scand., 64: 709-717, 1975.

11. Welch, J.K. and May, J.T. Anti-infective properties of breast milk. J. Pediatr., 94: 1-9, 1979.

12. Ford, J.E., Law, B.A., Marshall, V.M.E. and Reiter, B. Influence of the heat treatment on human milk and some of its protective constituents. J. Pediatr., 90: 29-35, 1977.

13. Gibbs, J.H., Fisher, C., Bhattacharya, S., Goddard, P., and Baum, J.D. Drip breast milk: its composition, collection and pasteurization. Early Human Dev., 1: 227-245, 1977.

14. Liebhaber, M., Lewiston, N.J., Asquith, M.T. Olds-Arroyo, L., and Sunshine, P. Alterations of lymphocytes and of antibody content of human milk after processing. J. Pediatr., 91: 897-900, 1977.

15. Raptopoulou-Gigi, M., Marwick, E., and McClelland, D.B.L. Antimicrobial proteins in sterilized human milk. Br. Med. J., 1:12-14, 1977.

16. Laurell, C.B. Electrophoretic and electro-immunochemical analysis of proteins. Scand. J. Clin. Lab. Invest., Suppl. 29: 1-136, 1972.

17. Bennett, R.M. and Mohla, C. A solid phase radioimmunoassay

for the measurement of lactoferrin in human plasma: variation with age, sex and disease. J. Lab. Clin. Med., 88: 156-166, 1976.

18. Goldblum, R.M., Powell, G.K., and Van Sickle, G. Secretory IgA in the serum of infants with obstructive jaundice. J. Pediatr., 97: 33-36, 1980.

19. Van Bavel, J. and Goldblum, R.M. Immunoglobulin A production by human colostral cells: quantitative aspects. In: Secretory Immunity and Infection. J. R. McGhee, J. Mestecky, and J. L. Babb (ed). Plenum Publishing Corp., 1978, 107: 87-94.

20. Ahlstedt, S., Carlsson, B., Hanson, L.A., and Goldblum, R.M. Antibody production by human colostral cells. I. Immunoglobulin class, specificity and quantity. Scand. J. Immunol. 4: 535-539, 1975.

21. Engvoll, E. and Perlmann, P. Enzyme-linked immunosorbent assay, ELISA. III. Quantitation of specific antibodies by enzyme-labeled anti-immunoglobulin in antigen coated tubes. J. Immunol., 109: 129-135, 1972.

NUTRITION RESEARCH, Vol. 1, pp. 461-466, 1981
0271-5317/81/050461-06$02.00/0 Printed in the USA.
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NUTRITIONAL AND PULMONARY FUNCTION ASSESSMENT IN PATIENTS WITH CHRONIC OBSTRUCTIVE LUNG DISEASE

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ABSTRACT

This investigation was undertaken to determine the prevalence and degree of malnutrition among patients with chronic obstructive pulmonary disease and to determine if a relationship exists between nutritional assessment parameters and pulmonary function. Thirty-seven patients admitted for the treatment of chronic obstructive pulmonary disease to a respiratory care unit of a Veterans Administration facility were studied. Nutritional status was evaluated using anthropometric measurements, laboratory data and immunologic assay. Deficits in anthropometric measurements were frequent and severe in this group of patients. Furthermore, our data demonstrated that patients with a forced vital capacity <30% of predicted value had significantly reduced lean body mass as measured by the mid-arm muscle circumference ($p < 0.05$). Investigation of the nutritional status of patients with chronic obstructive pulmonary disease using controlled nutrition intervention trials may elucidate the relationship of nutritional state to pulmonary function in this group of patients.

Key Words: Nutrition, pulmonary function, chronic lung disease

INTRODUCTION

We know of no previously published reports relating nutritional assessment parameters to pulmonary function in patients diagnosed with chronic obstructive pulmonary disease (COPD). However, nutrient intake studies of this patient population have been reported. In a study by Swartz et al of a group of active and retired coal miners with respiratory insufficiency, inadequate dietary intakes of calcium, vitamin A, vitamin C, thiamine and riboflavin were documented (1). Wilson et al calculated the nutrient intakes of a group of patients with emphysema and demonstrated that the degree of respiratory insufficiency measured by the forced expiratory volume was directly related to total caloric intake (2). The most severely dyspneic patients had the lowest caloric intakes.

It has been our general clinical experience to observe cachexia in patients with COPD (3). This pilot study was undertaken to see if a relationship exists between nutritional assessment parameters and pulmonary function.

METHODS

Thirty-seven patients (age range 46-90 years) were admitted for treatment of COPD to a chronic care facility, the Veterans Administration Medical Center, Fort Howard, Maryland. Their nutritional status was evaluated using anthropometric, biochemical, and immunologic nutritional assessment parameters by a team

of three physicians, two dietitians, and a nurse. Specifically, height, weight, triceps skinfold, mid-arm muscle circumference, serum albumin, total lymphocyte count, skin reactivity to common antigens, and a derived transferrin level were determined.

Height and weight were obtained on each patient and the ideal body weight was derived from ideal weight tables (4,5). The percent ideal body weight (% IBW) for each patient was then calculated. Triceps skinfold (TSF) thickness was measured by a dietitian using Lange skinfold calipers (Cambridge Scientific Instruments, Cambridge, MD). The TSF thickness and mid-arm muscle circumference (MAMC) were measured by established techniques (5). The standards for TSF and MAMC used were derived from the age and sex specific American standards compiled by Frisancho in 1974 (6). The criteria for moderate or severe deficits in anthropometric, biochemical, and immunologic measurements are defined in Tables I and II.

TABLE I

Criteria Used for Defining Degree of Deficit in Anthropometric Measurements

	Moderate (% Standard)	Severe
IBW*	80-90%	<80%
TSF**	40-60%	<40%
MAMC**	80-90%	<80%

* Standards used are Metropolitan, ideal body weight corrected to nude weight without shoe heels (4,5).

** Standards used are United States standards (50th percentile data) from the Ten State Nutrition Survey: TSF-male=11mm; female=19mm: MAMC-male=27.0cm; female=21.3cm (6).

TABLE II

Criteria Used for Defining Severity of Biochemical and Hematologic Deficits

	Normal Range	Moderate Deficit	Severe Deficit
Serum albumin (g/dl)	<3.5	2.8-3.0	<2.8
Serum transferrin+ (mg/dl)	>200	160-180	<160
Total lymphocyte count	>1800	900-1500	<900

\+ Serum transferrin value derived from formula - (0.8 x total iron binding capacity) - 43.

Skin test antigens consisting of Candida, intermediate strength tuberculin (purified protein derivative), and mumps were injected intradermally according to standard techniques (7) on the forearm of patients not taking immunosuppressives and the reactions recorded at 48 hours. Anergy was defined as <5mm induration for all three antigens.

All patients underwent tests for forced vital capacity (FCV), one-second forced expiratory volume (FEV_1), and their respective ratio (FEV/FVC). The FVC was measured with a Wright Respirometer (British Oxygen Co., Cleveland, OH) and the FEV_1 was measured with a Hewlett Packard Analyzer, Model No. 1600 (Hewlett Packard, Palo Alto, CA).

Additional data recorded from patients' charts included blood gases, prescribed oxygen (L/min), respirator therapy and the number of prescribed drugs. Statistical methods used were the unpaired Student's and chi square tests. The level of significance was taken at a level of p value of ≤ 0.05.

RESULTS

Table III summarizes the characteristics of the patient population included in this study.

TABLE III

Age, Number of Drugs, Anthropometric Measurements, and Laboratory Data Among Patients with Chronic Lung Disease*

Age (years)	64 ± 10	(N=37)
Drugs (#)	8 ± 2	(N=37)
TSF (mm)	10 ± 6	(N=34)
MAMC (cm)	22.2 ± 3.7	(N=34)
Albumin (g/dl)	3.7 ± 0.5	(N=36)
Transferrin (mg/dl)	209 ± 33	(N=29)
TLC (cells/mm3)	2046 ± 1002	(N=37)

* Data expressed as mean ± SD.

Chart review revealed ten patients with congestive heart failure (three of whom had cor pulmonale), seven patients with ischemic heart disease and one patient had sinus tachycardia without heart failure.

The extent of moderate and severe deficits in anthropometric measurements in these patients is summarized in Table IV. It can be seen that the anthropometric measurements showed the most severe deficits. In one-fifth of the patients moderate deficits in serum albumin or transferrin were present, but severe deficits were uncommon.

TABLE IV

Percentage of Total Study Population with Moderate and Severe Deficits in Various Nutritional Parameters

	Severe	Moderate
Ideal body weight	42	16
Triceps skinfold	29	9
Mid-arm muscle circumference	26	37
Serum albumin	3	6
Serum transferrin	3	14
Total lymphocyte count	8	30

Moderate and severe lymphocytopenia were present in 30 and 8 percent of patients, respectively. The results of delayed hypersensitivity skin reactions to common antigens showed seven of nine patients tested to be anergic to all three antigens. Because of the high prevalence of anergy, enumeration of the lymphocyte population was performed in nine patients. Although the number of B-lymphocytes was normal in all patients, the number of T-lymphocytes was low in eight of the nine patients. In this group the mean percentage of peripheral blood lymphocytes forming spontaneous rosettes with sheep erythrocytes was 27% (normal range in our laboratory, 46-68%).

In order to relate pulmonary function to nutritional assessment parameters, patients were separated according to the percentage of their predicted values for the FVC and FEV_1. Values were arbitrarily used to separate patients into groups. Nutritional and therapy parameters were then analyzed for the various groups. Although there was a tendency for low FEV_1 values to reflect low body weights and MAMCs, the relationship was not statistically significant. Patients with a low FVC demonstrated greater deficits in ideal body weight, MAMC and serum albu-

min (Table V). However, only the deficit in MAMC in the group with a FVC of <30% of predicted was statistically significant ($p < 0.05$) when compared to the group with a FVC of >30%. The patients with a FVC <30% were also prescribed more medication and all were receiving oxygen therapy.

TABLE V

Comparison of Nutritional Assessment and Treatment Parameters (mean ± SD) Between Patients with a FVC of <30% and >30% of the Predicted Value

Patients	FVC <30% (N=9)	FVC >30% (N=28)	Significance
TSF (mm)	13 ± 2	10 ± 6	NS
MAMC (cm)	20.1 ± 1.5	23.0 ± 3.7	$p < 0.05$*
Albumin (g/dl)	3.6 ± 0.5	3.8 ± 0.5	NS
Transferrin (mg/dl)	240 ± 41	204 ± 31	NS
TLC (cells/mm^3)	2191 ± 1577	2101 ± 892	NS
Drugs (#/patient)	9 ± 2	7 ± 2	NS
Oxygen therapy (# of patients)	9	12	NS

* Unpaired Student's test.

DISCUSSION

In spite of the availability of nutritional surveys of a general nature, there are few studies which provide data that permit a comparison of the nutritional status of patients in various disease categories. Information of this type can be valuable since it can identify patient groups in special need of nutritional support and may be of value in predicting the clinical course in certain situations.

In 1974, Butterworth pointed to the extent of malnutrition in hospitalized patients (8), and his findings were subsequently confirmed by other investigators. Willard et al assessed the protein caloric status of 200 adult non-obstetric admissions and found 63 (31%) of the patients to be malnourished (9). In his report the most common conditions associated with decreased protein stores were hypoxic cardiopulmonary disease and gastrointestinal disease. In our medical center with a predominantly male patient population, thirty-four percent of 176 hospitalized patients were moderately or severely malnourished (3). Among all patients, those with cancer and pulmonary disease had the greatest prevalence of malnutrition.

Our study of relatively stable patients with COPD shows a high prevalence of nutritional deficits, particularly in anthropometric measurements. Statistical analysis showed a significant relation ($p < 0.05$) between forced vital capacity and lean muscle mass as measured by the mid-arm muscle circumference. The choice of a FVC <30% was based upon our clinical experience. This group included patients with severe functional (exercise) disability, all of whom were receiving chronic oxygen therapy. At a FVC >40% and >50% of predicted value there was no significant correlation with the MAMC. The relationship between the FEV_1 and the MAMC did not reach statistical significance. However, the FEV_1 may be normal in patients with restrictive lung disease, whereas the FVC is abnormal in patients with either restrictive or obstructive lung disease. Although our study was limited in scope, a number of clinical variables were considered. Almost one-third of the patients had congestive heart failure and about one-half of the patients received chronic oxygen therapy. There was a definite trend for patients with low FVC to receive oxygen (Table V) but this group was not distinguishable in terms of nutritional parameters.

Thurbleck (10) reported that patients with emphysema had a tendency to be

underweight. He further showed that diaphragmatic weight was diminished in these patients at necropsy and that diaphragmatic weight correlated significantly with body weight ($p < 0.05$). Similarly, a report by Butler described diminished diaphragm muscle area in patients with emphysema (11). Recently, Moran et al showed a significant correlation between respiratory muscle strength and each measure of skeletal muscle function (12). These findings, as well as ours, suggest that malnutrition and consequent muscle wastings may add to the respiratory embarrassment of patients with COPD since in these patients airway obstruction increases the work of breathing.

From a clinical viewpoint, it is not surprising that weight loss is prevalent among COPD patients. Many of these patients receive medications such as digitalis, xanthine derivatives or other bronchodilators which have high anorectic potential. A substantial proportion of these patients fall within an elderly age group which is especially susceptible to malnutrition (13,14). Emotional factors may also be of considerable importance in a chronic disease process punctuated by acute exacerbations. Indeed, depression, anxiety and cyclic mood fluctuations are commonly seen in COPD patients (15). Lastly, the degree of subjective dyspnea among emphysematous patients has been related to poor intake (2).

A somewhat unexpected finding in this study was the presence of anergy in a substantial proportion of COPD patients tested with common skin antigens. Many of these patients had a significantly reduced number of circulating T-lymphocytes. However, Law et al, as well as others, have reported depression of both T- and B-lymphocyte systems in protein caloric malnutrition (16,17). Although the significance of a depressed delayed hypersensitivity response in COPD patients is not known, some authors have pointed to the prognostic value of anergy in other clinical settings (18).

Our data suggest the need for a comprehensive study of patients with chronic obstructive pulmonary disease where nutritional and detailed pulmonary function measurements can be determined. A controlled nutrition intervention trial is indicated to see if the anorexia and cachexia observed in COPD patients can be interrupted and to determine the type and extent of improvement in pulmonary function after nutritional supplementation.

REFERENCES

1. SWARTZ, S., KONISHI, F., and SANJABI, P. Dietary assessment of coal miners in southern Illinois. Am. J. Clin. Nutr. 3:1465-1468, 1978.

2. WILSON, N., WILSON, R., and FARBER, S. Nutrition in pulmonary emphysema. J. Am. Diet. Assoc. 64:530-536, 1964.

3. BUSHMAN, L., RUSSELL, R., WARFIELD, L., CURRY, G., and IBER, F. Malnutrition among patients in an acute care veterans facility. J. Am. Diet. Assoc. 77:462-465, 1980.

4. JELIFFE, D.B. The assessment of nutritional status of the community. WHO, Geneva, 1966.

5. BLACKBURN, G.L., BISTRIAN, B.R., MAINI, M.S., SCHLAMM, H.T., and SMITH, M.I. Nutritional and hospital assessment of the hospitalized patient. J. Parent. Enter. Nutr. 1:11-22, 1977.

6. FRISANCHO, R. Triceps skinfold and upper arm muscle size norms for assessment of nutritional status. Am. J. Clin. Nutr. 27:1052-1058, 1974.

7. SPITLER, L.E. Delayed hypersensitivity skin testing. In: Manual of Clinical Immunology. N. R. Rose and H. Friedman (eds.), Am. Soc. Microbiol., Washington, D.C., 1976, pp. 53-63.

8. BUTTERWORTH, C.E. The skeleton in the hospital closet. Nutr. Today 9:4-8, 1974.

9. WILLARD, M.D., GILSDORF, R.B., and PRICE, R.A. Protein-calorie malnutrition in a community hospital. JAMA 243:1720-1722, 1980.

10. THURBLECK, W. Diaphragm and body weight in emphysema. Thorax. 33:483-487, 1978.

11. BUTLER, C. Diaphragmatic changes in emphysema. Am. Rev. Respir. Dis. 114:155-159, 1976.

12. MORAN, L.R., THURLOW, J., CUSTER, P., and GRANT, J.P. Nutritional assessment as a predictor of physical performance. Aspen 4th Clinical Congress-The Art and Science of Nutrition, 62, 1980.

13. JUSTICE, C.L., HOWE, J.M., and CLARK, H.E. Dietary intakes and nutritional status of elderly patients. Study in a private nursing home. J. Am. Diet. Assoc. 65:639-646, 1974.

14. O'HANLON, P., and KOHRS, M.B. Dietary studies of older Americans. Am. J. Clin. Nutr. 3:1257-1269, 1978.

15. DUDLEY, D., WEMUTH, D., and HAGUE, W. Psychological aspects of care in the chronic obstructive pulmonary disease patient. Heart and Lung 2:389-393, 1973.

16. LAW, S.K., DUDRICK, S.J., and ABDON, N.I. Immunocompetence of patients with protein-calorie malnutrition. The effects of nutritional repletion. Ann. Intern. Med. 79:545-550, 1973.

17. DOUGLAS, S.D., and FAULK, W.P. Immunological aspects of protein calorie malnutrition. In: Recent Advances in Clinical Immunology. R.A. Thompson (ed.), Churchill Livingstone, New York, 1977, pp. 15-39.

18. PIETSCH, J.B., MEAKINS, J.L., and MACLEAN, L.D. The delayed hypersensitivity response. Application in clinical surgery. Surgery 82:349, 1977.

NUTRITION RESEARCH, Vol. 1, pp. 467-488, 1981
0271-5317/81/050467-22$02.00/0 Printed in the USA.
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STUDIES ON PROTEIN AND ENERGY REQUIREMENTS IN CHINA*

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ABSTRACT

Protein and energy requirements of different population groups in China have been studied. Together with the data from other countries, Recommended Dietary Allowances were established and were revised in 1962 and again in 1976. The average BMR of 19 male subjects aged 20-30 was 37.8 kcal/min/m^2 of body surface. The average energy expenditure of 16 male college students was 2420 kcal/day, and the energy absorbed was 2976 kcal/day. The energy expenditure of 6 female students was 2170 kcal/day. There was a wide variation of energy expenditure among workers and peasants according to the intensity of work. From dietary surveys on the senior middle school boys, the energy intake of adolescents was 2618-3938, which is near or above the RDA. The calorie intake of 8 infants in a metabolic study was 100-200 kcal/kg/day. From nitrogen balance studies, the daily protein requirements of Chinese young adults and infants under 6 months of age consuming soybean milk substitute were determined to be 1.0 g/kg, and 4.5 g/kg respectively. Dietary surveys showed that the protein intake of children with 2-3 years of age was about 30-40 g/kg/day.

The traditional Chinese diet with high plant protein and crude fiber content may be beneficial in preventing coronary heart disease. However, the question of absorption and utilization of protein and energy requires further investigation. The effect of meal frequency in relation to the health of the student has been studied, but further study is necessary. Based on our experience over the last 30 years, food rationing determined by age and physical activity appears to be the most efficient method of fighting hunger and malnutrition.

KEY WORDS : Protein requirements, Energy requirements, Infant nutrition.

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* Presented at the Workshop on Protein-Energy Requirements, United Nations University/International Union of Nutritional Sciences, Cambridge, Mass. U.S.A., May 19-23, 1980.

Recent advances in our knowledge of vitamins and trace elements have pushed energy and protein metabolism into the background. However, in large areas of the world there is not enough protein to meet the needs of the people, and caloric requirements are still the first consideration in nutrition for most of the people in the world. Two-thirds of the world's population subsists entirely on rice, corn, wheat, or casava. Because the developing countries have few animal proteins available, it is important for researchers to try to develop products that will provide high plant protein and also to determine the minimal amount of good quality that will meet adequately the people's needs. Freedom from want means, first of all, freedom from hunger. Freedom from hunger demands a sufficiency of calories. The first aim of the "World Hunger Program" is to assure freedom from hunger for all the people in the world and to maintain their good health. Since 1949, nutritionists of the People's Republic of China have undertaken the following areas of research in this field.

RECOMMENDED DIETARY ALLOWANCES

The purpose of the RDAs for our Chinese population is to provide the requirements for individual variations among most persons as they live in China under normal environmental stress (1,2). It is believed that the RDAs will maintain good nutrition in practically all healthy persons in China. Some nutritionists believe that the recommendations exceed actual needs, and so they accept three-fourths or even two-thirds of the RDA values as adequate.

The 1973 FAO/WHO energy recommendations, however, have taken a major step by allowing for differences in physical activities and body weights of both men and women, and include the energy needs for those who are slightly active, moderately active, very active, and exceptionally active. The total energy metabolism of a person is known to depend on a number of factors, i.e., age, body weight, external temperature, and activity. With the exception of activity, the relationship between energy metabolism and each of these factors can be expressed in simple terms (3).

Sex and weight: $E = 152W^{0.73}$ for man and $E = 123W^{0.73}$ for woman, where E = energy metabolism in calories and W = body weight in kg.

Age: After the age of 39 years, the FAO/WHO recommends a decrease in the energy intake for the 20-39 year age group, a 5% decrease for the 40-49 year age group, a 10% decrease in the 50-59 year age group, a 20% decrease for the 60-69 year age group, and a 30% decrease for the 70-79 year age group. The FAO/WHO energy requirements for children and adolescents, assessed independently of the reference adults, have been determined according to body size only.

External temperature: Increase of temperature decreases the energy metabolism. Every 10°C rise in temperature causes a 5% decrease of energy metabolism. All these values are based on the best data available and are given in round numbers (Table 1).

Dietary protein provides amino acids which are required for the body to build and maintain tissues; to form enzymes, some hormones, and antibodies; to regulate certain body processes; and to produce energy. The need for

Table 1. Recommended Daily Dietary Allowances

		Calories (kcal)	Protein (g)
Men (65 kg)	Sedentary work	2400	70
	Light work	2600	75
	Moderate work	3000	80
	Heavy work	3600	90
	Very heavy work	4200	105
Women	Sedentary work	2200	65
	Light work	2400	70
	Moderate work	2600	75
	Heavy work	3400	85
	Pregnancy (2nd half)	+ 300	+15
	Lactation	+1000	+25
Adolescents			
Boys (54 kg)	16-19 yr.	3000	90
Girls (50 kg)	16-19 yr.	2700	80
Boys (42 kg)	13- yr.	2600	80
Girls (42 kg)	13- yr.	2500	75
Children	10- yr.	2300	70
	7- yr.	2000	60
	5- yr.	1600	50
	3- yr.	1400	45
	2- yr.	1200	40
	1- yr.	1100	40
	7-12 mo.	100/kg	3.5/kg
	0-6 mo.	120/kg	4 5/kg

dietary protein to build new tissue and to maintain existing tissue continues throughout life. The total quantity of protein needed for maintenance (expressed as the amount/kg of body weight) increases with age until a fairly constant level is reached at approximately 20 years of age; the protein required decreases with the age beginning at the age of 20.

The FAO/WHO Committee on Energy and Protein Requirements developed what is called "Safe Level of Intake of Protein" for children, adults, and pregnant and lactating women. Using the factorial method, the Committee determined the losses of nitrogen (protein) laid down during growth, pregnancy, or lactation. The protein values that were found to meet the losses incurred and to provide for the formation of new tissue were increased by 30%; the increased values were called the "physiological nitrogen requirement". The committee added 30% to the physiological requirements to allow for individual variations and thus arrived at its recommendations.

The FAO/WHO recommendations take into account the quality of the protein in the diet; values are given for diets having protein qualities of 60%, 70%, and 80% relative to milk (amino acid score, 95) and eggs (amino acid score, 100). The committee assumed that the diets of people in relatively rich countries have a protein quality of about 80%, while the diets of those people in relatively poor countries would have about 70%. In some situations where 70-80% of the dietary protein comes from casava and corn and virtually none from animal foods, the relative protein quality may be as low as 60% (Table 2).

ENERGY REQUIREMENTS

Calorie requirements may be assessed in two ways: 1) Measurement of heat production. The amount of heat produced by the human body is measured directly in a respiration calorimeter or calculated from the amount of carbon dioxide produced and the amount of oxygen consumed. 2) Measurement of food consumption. When the body weight and composition remain unchanged, the amount of heat produced is equal to the caloric value of the food consumed minus the caloric value of the products excreted.

In the first method, the daily heat production may be broken down into several components for different periods of the day, corresponding to sleeping, walking, eating, dressing, etc. The daily heat production is broken down even more simply into two components: basal metabolism, minimum heat production for maintenance of life, and work, additional heat production due to voluntary muscular activity.

In the second method of assessing calorie requirements, knowledge is required of the composition of foods, coefficient of digestibility, and degree of utilization of the major food constituents in each kind of food. Such knowledge is very limited at present.

In view of all the uncertainties in estimating the actual heat production and the calorie value of food consumed, it is not surprising that deficiency or excess of the order of 10% or more is a matter of common occurrence.

Adults

Basal metabolism refers only to the amount of energy needed for vital life processes, i.e. the amount of heat given off by an individual during physical, digestive, and emotional rest. Any kind of physical activity increases the energy need above the basal metabolism. Different activities require different amounts of energy. An indirect way of measuring the energy

Table 2. FAO/WHO (1973) Recommended levels of Protein Intake

	Body weight (kg)	Recommended level of protein intake		Adjusted level for protein of different quality (g/person/day)		
		Grams protein per kg body wt. per day	Grams protein person per day	Score* 80	Score 70	Score 60
Adult men	65.0	0.57	37	46	53	62
Adult women	55.0	0.52	29	36	41	48

* Scores are estimates of the quality of protein usually consumed relative to that of egg or milk (Ideal, Score 100). If the relative quality is 80, the adjusted level of intake for adult man would be $\frac{37 \times 100}{80}$ = 46 g.

used for a particular activity was the Douglas method. The expired air of the individual performing the activity was collected in a large bag and then analyzed for carbon dioxide and oxygen.

(1) ENERGY EXPENDITURE OF COLLEGE STUDENTS (4): The average BMR of 19 male subjects aged 20-30 was 37.8 kcal/min/m^2 of body surface. The energy cost of the major activities of medical students were determined by Douglas' method. Those activities were classified into sedentary, moderate, and heavy, and were expressed in kcal/min/m^2 (Table 3). The average expenditure of 16 male subjects was 2420 kcal/day. The protein expenditure of 6 female students was 2170 kcal/day. The non-protein respiratory quotient of 9 male subjects was determined, the the sources of energy consumed were: protein 12.3%, fat 28.8%, and carbohydrate 58.9%. The sources of energy absorbed from protein, fat, and carbohydrate contained in the diet were 12.0%, 27.9%, and 60.1%, respectively.

(2) ENERGY EXPENDITURE OF WORKERS (5-10): Tables 4 and 5 show the energy expenditure of persons engaged in iron and steel work. The Douglas bag technique was used, and this survey was carried out under normal industrial conditions in peace time. The energy expenditure was calculated over the whole shift by a combination of time studies and indirect calorimetry. Here it is expressed in kcal/min/m^2. It is obvious that generalizations from the iron and steel industry data are unjustified. There are a great variety of jobs and many forms of mechanization in different plants. Undoubtedly some workers do heavy work regularly; but in most modern plants many do light work only. There is a wide variation in the intensity of work, and men worked very hard for some periods of the working day. Some of the energy costs are not high. This was due in part to large differences between the nominal times for rest (by regulation) and the actual work times in effective work. The ratio between working hours and the rest period within the eight hours is usually about 6:4. These surveys recorded only the energy expenditure at work. The assessed dietary needs (an arbitrary figure for the caloric needs for non-occupational activities must be added (Table 4,5).

Body surface and energy expenditure of coal miners are shown in Tables 6 and 7, respectively, and Table 8 shows the cost of some typical activities. Although there is considerable variation in individual results, these data show a remarkable general agreement with the results of other countries (II). The gross energy expenditure in calories/min of working time varied from 2.9 to 3.7 with a mean of 3.3, and for the total time spent underground the figure varied from 1.8 to 2.3 with a mean of 2.1. Miners may spend from 30 min to two hours during every shift, walking over uneven surfaces up and down inclines. Stooping is often necessary owing to the low ceilings. It has been reported that the average cost of walking was increased by 28% with a half stoop and 65% with a full stoop. It is well known that the journeys to and from the coal face may be very hard work. Out of 17 types of work specified in Table 6, the cost of carrying a trestle in the wind tunnel and climbing in the wind tunnel showed an expenditure of 5.093 and 4.432 kcal/min/m^2, respectively. Although the basic mining process involves a high rate of energy expenditure, the overall rate of energy expenditure may be reduced by rest periods (average about 3 of the 8 h). Thus, daily rates of energy expenditure always may not be high.

Dietary surveys have been carried out on a large scale in many countries. The information obtained has been the basis of many estimates of human caloric requirements. It is relatively simple to carry out a family survey, but individual dietary surveys, in which all the foods consumed by a single person have to be measured, are technically more difficult, although they provide much more detailed information. Table 9 shows the energy and protein intakes

Table 3. Energy Expenditure of Different Activities of College Students

	Activity	No. of Subjects	Energy Expenditure (kcal/min/m^2 Mean(& range)
Sedentary	Lying at ease	22	0.652 (0.583 - 0.737)
	Sleep	6	0.732 (0.686 - 0.818)
	Snooze	7	0.781 (0.652 - 0.887)
	Rest between classes	2	0.786 (0.755 - 0.817)
	Sitting, watching movie	4	0.806 (0.755 - 0.839)
	Sitting, watching demonstration	5	0.808 (0.714 - 0.954)
	Sitting, writing	9	0.812 (0.730 - 0.918)
	Attend meeting	6	0.812 (0.737 - 0.872)
	Sitting, studying	30	0.830 (0.730 - 0.977)
	Sitting, reading	19	0.846 (0.719 - 1.084)
	Examination	5	0.916 (0.730 - 1.023)
	Writing on blackboard	1	0.980 (0.980 - 0.980)
	Standing and listening	2	0.985 (0.878 - 1.092)
	Working in laboratory	11	1.002 (0.710 - 1.190)
	Lying, reading	2	0.804 (0.790 - 0.819)
Moderate			
	Cleaning window	1	1.983 (1.983 - 1.983)
	Undressing	4	2.170 (2.060 - 2.280)
	Dressing	4	2.232 (2.232 - 2.232)
	Making bed	2	2.263 (2.210 - 2.325)
	Washing clothes	2	2.362 (2.170 - 2.555)
	Standing, conduct signing	2	2.645 (2.370 - 2.920)
	Ordinary morning drill	4	2.654 (2.215 - 3.800)
	Cleaning floor	2	2.716 (2.620 - 2.813)
	Walking	11	2.703 (2.330 - 3.302)
	Broadcast drill	2	2.770 (2.560 - 2.980)
	Scrubbing floor	1	2.817 (2.817 - 2.817)
Heavy			
	Dancing	3	4.031 (3.307 - 4.805)
	Playing baseball	2	4.032 (3.913 - 4.150)
	Playing volleyball	2	4.072 (3.820 - 4.325)
	Running	2	5.302 (3.853 - 6.750)
	Playing basketball	6	5.785 (5.000 - 7.916)
	Playing football	2	5.965 (5.330 - 6.600)

Table 4. Energy Expenditure in Iron and Steel Work

Type of work	Working time/day (hr)	Working time (%)	Rest time (%)	Kcal/min/m^2	Kcal/min
Furnaceman	8.00	55.09	44.91	1.864	2.9263
Ore carrying worker	8.00	71.85	28.15	3.066	5.2858
Ore sieving worker	8.00	59.79	40.21	2.713	4.9764
Blast furnaceman	8.00	72.71	27.29	2.972	4.8265
Sintering (non-mechanized)	8.00	70.50	20.50	2.696	4.4214
Open-hearth furnace	8.00	42.00	58.00	1.770	2.8426
Electric open-hearth	8.00	39.77	60.23	1.780	2.8694
Coke over man (mechanized)	6.50	69.90	30.10	1.958	2.9507
Coke oven man (semi-mechanized)	7.66	60.38	39.62	1.602	2.4126
Rolling worker	8.00	55.27	44.73	1.665	2.7139
Rolling worker	8.00	45.93	54.06	1.527	2.3668
Tube casting foundry work					
Moulder	8.05	74.74	25.26	1.609	2.5857
Core layer	8.33	45.80	54.20	1.404	2.2576
Fettling work	8.94	58.96	41.04	1.925	2.8976
Fettling work	8.83	62.84	37.16	1.884	3.2194
Chassis making	7.55	74.40	25.60	2.128	3.4091
Core removal	8.30	81.53	18.47	1.930	3.1015

Table 5. Energy Expenditure of Different Activities in Iron and Steel Work

Activities	No. of subjects	Energy Expenditure Cal/min/m^2
Iron Work		
Crushing iron with 10 kg hammer	5	3.306 ± 0.094
Carrying 100 kg iron with another person	8	2.927 ± 0.0927
Slag removal	7	2.460 ± 0.102
Moulding	9	2.029 ± 0.082
Open-hearth furnace		
Iron crust removal	8	4.547 ± 1.013
Shovelling and throwing refractory sands	9	4.374 ± 1.674
Sending refractory sands with a 39 kg iron spoon	10	3.134 ± 1.142
Miscellaneous work	14	3.090 ± 0.888
Blowing compressed air into the oven	11	2.999 ± 0.857
Packing the orifice of oven	11	2.992 ± 0.946
Cleaning the orifice of oven	6	2.782 ± 0.793
Sampling	9	2.525 ± 0.771
Walking	6	1.706 ± 0.342
Rest	7	0.918 ± 0.200
Electric open-hearth		
Slag removal	9	4.537 ± 1.322
Sending refractory sands with a 39 kg iron spoon	8	4.057 ± 0.711
Shovelling and throwing	9	3.827 ± 0.806
Sampling	5	3.719 ± 0.893
Miscellaneous	10	3.406 ± 0.494
Packing the orifice of oven	7	3.294 ± 0.470
Pushing 400 kg steel ingot into oven	9	2.383 ± 0.636
Rest	9	1.079 ± 0.230
Coking work		
Shovelling coke onto conveyor belt	10	3.756 ± 0.629
Cleaning oven	9	3.364 ± 0.604
Open oven door with 3-5 kg iron stick	10	2.992 ± 0.853
Pushing coke	4	2.976 ± 0.616
Cleaning coke residuel	4	2.855 ± 0.962
Pushing coal	9	2.751 ± 0.661
Shutting oven door	3	2.047 ± 0.280
Opening oven door	7	2.033 ± 0.331
Cleaning coal (mechanized)	6	3.029 ± 0.668
Closing oven door (mechanized)	6	2.886 ± 0.678
Cleaning oven (mechanized)	6	2.887 ± 0.592
Rest	5	0.780 ± 0.082
Rolling work		
Hand rolling	13	2.519 ± 0.140
Hand rolling	7	2.358 ± 0.159

Table 5. (cont'd).

	Hand rolling (heavy)	3	4.928 ± 0.133
	Bundling steel rod	4	4.396 ± 0.386
	Rolling the reinforcing bar onto the wheel	4	3.402 ± 0.355
	Bundling the reinforcing bar with wire	4	2.212 ± 0.306
	Miscellaneous	8	2.058 ± 0.198
	Hand rolling (first step)	14	2.762 ± 0.149
	Hand rolling (second step)	6	1.877 ± 0.157
Moulding work			
	Rolling chassis	10	3.419 ± 0.671
	Handling chassis	9	3.159 ± 0.44
	Fettling core	8	2.708 ± 0.687
	Pile up the chassis	9	2.676 ± 0.649
	Making chassis	6	2.753 ± 0.297
	Core removal	4	2.554 ± 0.546
	Fettling core	8	2.554 ± 0.801
	Hammer	10	2.420 ± 0.430
	Wrapping paper	11	2.365 ± 0.316
	Cleaning core	4	2.339 ± 0.457
	Planning core	10	2.288 ± 0.383
	Rolling tubes	9	2.146 ± 0.623
	Moulding	4	1.787 ± 0.040
	Putting core into the mold	4	1.667 ± 0.433

Table 6. Body Surface of Coal Miners

Type of work	Mining	Tunnelling	Timbering
Age	30 (21-42)	27 (18-36)	32 (21-46)
Height (cm)	164.2 (153.5-172.2)	166.6 (156.8-171.2)	164.5 (152.4-170.7)
Body weight	55.4 (48.2-62.0)	58.6 (55.2-66.0)	57.8 (49.1-67.7)
Body surface (m^2)	1.559 (1.402-1.703)	1.614 (1.509-1.736)	1.604 (1.409-1.749)
No. of subjects	19	9	7

Table 7. Energy Expenditure During Coal Mining

Type of work	Working time (min)	Total energy cost (kcal/min/m^2)	Body surface (m^2)	Total energy (cost (kcal/8h/man)	Pure working time (kcal/min)
Coal miner	480 (209*)	2.212	1.559	1655.1	3.448
Coal timbering worker	480 (209)	2.275	1.559	1702.9	3.548
Coal drilling worker	480 (173)	1.930	1.559	1444.9	3.010
Blasting worker	480 (88)	2.348	1.559	1756.7	3.658
Tunnelling worker	480 (200)	1.811	1.614	1403.2	2.923
Rock transferring worker	480 (249)	1.954	1.914	1513.6	3.633
Timbering worker	480 (177)	2.088	1.604	1607.7	3.350

* indicates rest time in minutes within 8 hours.

Table 8. Energy Expenditures of Different Activities During Coal Mining

	Type of work	No. of Subjects	Energy Expenditure kcal/min/m^2
Coal mining			
	Hewing coal	8	3.143 ± 0.837
	Timbering	9	3.882 ± 1.321
	Chopping log	7	2.760 ± 0.565
	Walking with TNT on shoulder	4	2.165 ± 0.701
	Carrying trestle in the wind tunnel	5	5.093 ± 0.650
	Drilling coal	10	1.805 ± 0.318
	Packing hole with TNT	6	1.601 ± 0.651
	Pushing scraper-trough conveyer	5	2.771 ± 0.640
	Climbing wind tunnel	7	4.432 ± 0.940
	Walking in the main tunnel	5	2.382 ± 0.862
	Miscellaneous work	5	2.678 ± 0.682
Tunnelling timbering			
	Drilling rock	9	1.752 ± 0.677
	Pushing wheelbarrow loaded with stone	9	3.723 ± 1.186

Table 8. (Cont.)

	Type of work	No. of Subjects	Energy Expenditure kcal/min/m^2
	Carrying rail trestle	6	2.593 ± 0.635
	Carrying stone by hand	6	2.637 ± 0.977
	Setting cement plate	5	3.410 ± 0.811
	Mixing mud	6	2.913 ± 0.207
Resting in sitting position		6	1.073 ± 0.176

Table 9. Dietary Proteins and Energy Intake of Workers

Type of work	Calories (Kcal)	Protein	Location	Place
Iron and steel work	4100	125	Wu-han	Board
Iron and steel work	3956	125	Wu-han	Home
Iron and steel work	3368	110	Hu-nan	Board
Iron and steel work	3921	132	An-shan	Board
Electric power industry	3112	105	Chong-qing	Board
Coal mining	3713	96	An-hui	Board
Iron factory	3347	80	Hang-zhou	Board
Machine factory	3168	86	Guang-si	Board
Silk plant	2885	89	Hang-zhou	Board
Building worker	3614	108	Peking	Board
Mining	3950	86	Kwang-tong	Board

Table 10. Energy Expenditure of Male Peasants

Type of work	No. of Subjects	cal/min/m^2
Mowing wheat	3	2.7896 ± 0.2011
Light plowing	6	2.9368 ± 0.1783
Carrying water with tubs	6	3.1943 ± 0.1463
Shovelling earth	5	3.5069 ± 0.0824
Planting field	4	3.9133 ± 0.3455
Transplanting rice seedlings	4	2.2803 ± 0.3138
Weeding the seedling field	4	2.7188 ± 0.3334
Weeding rice field	5	3.3326 ± 0.5386
Weeding rice field (deep)	3	4.1167 ± 0.4174
Walking	4	2.1319 ± 0.3852
Rest	2	1.0203 ± 0.0000

of workers in some provinces of China. "Intake must be equal to output", but the application of this principle requires knowledge of details which are still lacking. There is wide variation in the caloric requirements on individuals, even of those having the same occupation and leading similar kinds of lives.

(3) ENERGY EXPENDITURE OF PEASANTS (12). Measurements of the energy expenditure of peasants in agricultural work have been made in China, and the data are shown in Tables 10-14. Time studies were performed and an estimation of total daily expenditure was made. The values for energy expenditure for men who worked long hours in the summer harvest and spring digging were 4538 and 4954 kcal, respectively. In these studies the men worked with little or no mechanically powered equipment (Tables 10-14).

Adolescents and Children

We have no data on the energy expenditure of adolescents, but from a dietary survey on the senior middle school boys (13-14), the energy intake of adolescents was 2618-3938 kcal, which is near to or above the RDA. The data obtained from our metabolic study on soybean milk substitute with infants (37-77 days of age at the start and 6-7 months at the end) revealed that there were no significant differences between the availability of energy from the soybean product and the powdered cow's milk control diet; the former was 91.2% while the latter was 92.7%. The calorie intake of 8 infants was almost identical at 100-200 kcal/kg/day (15). The slopes of their growth curves are nearly the same. However, the slope of the growth curve of one baby whose calorie intake never reached 100 kcal/kg/day, is evidently much flatter than the slope of the growth curves of the other infants. These results once more confirmed that 100-120 kcal, with at least 15% from protein, should be supplied to infants for optimal growth (Table 15).

PROTEIN REQUIREMENTS

Obviously protein requirements differ from person to person and depend on many factors, e.g., the daily turnover of protein within the body, the ability to convert one amino acid to another, and the particular needs for certain enzymes and hormones. Some pure amino acids became available on a large scale for experimental purpose, and it was possible to feed human beings on artificial diets in which a mixture of amino acids was the sole source of nitrogen. If one of the essential amino acids was omitted from the mixture, an adult subject ceased to maintain nitrogen equilibrium. The missing amino acid was replaced in gradually increasing amounts, until the subject was again in nitrogen balance or until normal growth was resumed. In this way an estimate of the human requirements for each of the amino acids can be made. Such experiments are difficult and require much care and patience, and consequently the study protein of requirements using this method has its limitation. For practical purposes, a natural well-balanced diet is still the best choice.

The nitrogen balance method expressed the biological value as the percentage of the absorbed dietary nitrogen that is retained when the protein under investigation forms the only source of nitrogen in the diet. Using this method, the protein requirement of an individual can be estimated.

Adults

Wang and his colleagues (16) have carried out two nitrogen balance studies of protein requirements of Chinese young adults. In the first experiment when calorie intake was maintained at 2270-2379 kcal and protein intake at

Table 11. Energy Expenditure of Female Peasants

Type of work	No. of Subjects	Energy Expenditure Cal/min/m^2
Mowing wheat	11	1.7939 ± 0.0643
Pulling up wheat	3	3.2967 ± 0.2109
Spreading fertilizer (stand)	3	1.6172 ± 0.2195
Spreading fertilizer (stoop)	2	3.0406 ± 0.2075
Spreading manure	3	2.9522 ± 0.1386
Thinning young shoots	6	3.1553 ± 0.3277
Shovelling earth	3	3.4425 ± 0.1407
Planting field	2	3.8606 ± 0.6254
Weeding rice field	5	1.9116 ± 0.1554
Walking	5	1.9962 ± 0.2883
Rest	3	0.8850 ± 0.2761

Table 12. Energy Expenditure of Each Activity

Type of work	No. of Subjects	Energy Expenditure Cal/min/m^2
Carrying manure by shoulder	4	3.4985
Picking radishes with a hoe	2	2.9296
Raking the soil	3	2.7062
Picking corn roots with a hoe	4	2.5061
Spreading artificial manure	2	2.2986
Weeding with a hoe	2	2.0290
Picking potatoes with a hoe	14	1.9746
Binding corn stalks	2	1.8881
Mowing beans	9	1.8157
Picking corn	4	1.5745
Winnowing beans	5	1.5449
Pulling radishes by hand	4	1.5397
Loading corn onto carts	2	1.3007

Table 13. Distribution of Daily Time Unit: Hour

Type of work	Spring and Autumn		Summer		Winter		Summer harvest		Deep Digging	
	Part-time labor	Full time labor	Part-time labor	Full time labor	Part-time labor	Full time labor	Part-time labor	Full time labor	Part-time labor	Full time labor
Walking	1.0	1.5	1.0	1.5	0.5	1.0	1.5	1.5	1.0	1.0
Productive labor	7.5	8.25	6.0	7.0	6.0	7.0	9.0	11.5	8.25	10.75
Sleeping	8.5	8.5	9.0	9.0	10.0	10.0	8.5	6.5	9.0	7.5
Taking meal	1.5	1.5	1.5	1.5	1.0	1.0	2.0	2.0	2.0	2.0
Sewing	0.5	0.0	1.0	0.0	1.5	0.0	0.0	0.0	0.0	0.0
Cleaning street	0.25	0.0	0.25	0.25	0.25	0.25	0.25	0.0	0.25	0.0
Rest	1.0	1.0	1.0	1.0	0.5	0.5	1.0	1.0	1.5	1.5
Reading and attending meeting	1.5	1.5	1.0	1.0	2.0	2.0	0.0	0.0	0.0	0.0
Toilet	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Dressing and undressing	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Chatting	0.5	0.5	1.0	1.5	0.75	1.0	0.25	0.25	0.5	0.0
Washing clothes	0.5	0.0	1.0	0.0	0.25	0.0	0.25	0.0	0.25	0.0
Carrying water	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25

Table 14. Average Daily Energy Expenditure (Kcal) of Male and Female Peasants in Spring, Fall and Summer

Type of work	Average daily energy expenditure in spring & fall (Kcal/min/m^2)			Average daily energy expenditure in summer (Kcal/min/m^2)		
	Male	Female		Male	Female	
	Full-time labor	Full-time labor	Part-time labor	Full-time labor	Full-time labor	Part-time labor
Light ploughing	3898			3691		
Spreading		3408	3310		3310	3055
Thinning young shoots		3647	3445		3365	3102
Spreading fertilizer (stoop)		3563	3369		3437	3163
Planting field	4681	4189	3938	4356	3897	3558
Shovelling earth	4343	3886	3663	4069	3640	3337
Mowing				4538	3003	2740
Pulling (up) wheat					4568	4008
Deep digging	4954	4435	3812			

55.3-61.5 g/day (0.90-1.01 g/kg body wt.), the subjects were kept in slight positive nitrogen balance. In the second experiment, which was divided into 8 periods, the average calorie intake was 2664-3267 kcal. The protein intake for each period varied from 32 to 86 g. There were 9-10 subjects in each period. The experimental diet contained cereals, soybeans (or pork) and vegetables. The results showed that with either a vegetable diet or a mixed diet, there was a linear correlation between protein intake and nitrogen balance. According to this linear equation, the protein intakes needed for maintaining nitrogen equilibrium were 1.04 and 1.07 g/kg body weight, respectively. Thus, it may be concluded that a customary Chinese diet with an adequate energy supply and a protein intake of 1.0 g/kg body weight/day would satisfy the physiological minimum requirement.

There is much individual variation in the minimum amount of protein that must be fed in order to maintain nitrogen equilibrium. Most people maintain nitrogen balance on approximately 0.6 g/kg body wt/day of good quality protein for a long period. But in all prosperous societies adult intake of dietary protein is usually well above this level. As stated above, 1.0 g/kg body wt/day of our customary diet should provide the proper amount of protein for adult needs. From dietary surveys, the protein intake of Chinese workers was around 80-130 g/day (Table 9) (9,17) and of college and middle school students was approximately 74-115 g/day (13,14). These amounts are above the RDA. One might argue that a reduction of protein intake to the low level actually would be beneficial, because a high protein diet places a strain on the kidneys during the process of nitrogen excretion and tends to cause renal and vascular diseases. However, it is likely that in the developing countries there are large numbers of adults whose health is impaired by lack of adequate dietary protein, although there may be no obvious clinical signs of deficiency.

Children

Liu et al. (18) have carried out nitrogen metabolism studies on children 2-3 years of age. The results showed that there was no statistical difference in nitrogen retention between daily protein intake of 30 and 40 g when energy intake was approximately 1200 kcal/day. In our studies on soybean milk substitutes during 1954-57, metabolic experiments were carried out with 9 male infants (age 37-77 days at the start and 6-7 months at the end). These infants were fed with the soybean product and a cow's milk powder control in alternate periods, each of which lasted 2-4 wk. Some of the results are summarized in Table 15. It was found that when the average calorie intake was around 100 kcal/kg/day and the average nitrogen intake was 732 mg/kg/day during the soybean product period, the average nitrogen retention was 196 mg/kg/day.

Scheinberg's data (20) showing that infants taking 250-399 mg of nitrogen (1.56-1.99 g protein) from human milk retained 100-150 mg N/kg/day which seemed to be the suitable amount for promoting optimal growth under most natural conditions. Judging from the above figure, it may be concluded that an infant receiving 110 kcal and 732 mg of nitrogen (4.5 g protein) which results in an average retention of 196 mg N/kg/day would be adequate for those fed mainly with vegetable protein which furnished 15% of the total calories. Earlier studies (1928, 1930, 1931, 1940) showed the vegetable protein supplying 17-18% of the total calories, should be 5-6 g/kg/day (21).

From dietary surveys, the protein intake of the children 4-7 years of age in a county kindergarten was about 38 g (22) and children with 2-3 years of age in Peking nurseries was about 30-40 g/day (23) (Table 16).

Table 15. The Average Daily Caloric Intake and Utilization of Individual Infants in Different Dietary Periods.

Infants	CMP		SMS		SMS CLO		CMP CLO		SMS.HP CLO		Average	
	Intake Cal	Utilized %	Intake Cal	Utilized %	Intake Cal	Utilized %	Intake Cal	Utilized %	Intake Cal	Utilized %	Intake Cal	Utilized %
Ding	117	93.2	120	91.7	108	93.5	116	94.8	111	90.0	114	92.7
Liu-1	114	93.8	129	92.2	122	91.0	117	94.8	116	88.7	119	92.1
Chen	120	94.2	148	90.6	107	93.5	116	93.0	103	90.2	119	92.2
Liu-2	107	92.5	107	91.5	98	95.0	106	90.7	101	95.0	103	92.3
Chi	116	89.7	120	90.0	122	01.7	103	93.3	109	88.9	114	90.6
Wang	117	91.5	121	91.7	113	92.0	101	95.1	105	88.5	113	90.7
Zhang	113	89.4	108	91.7	105	92.4	107	93.5	110	89.0	108	93.2
Kwan	107	88.0	107	87.8	104	89.4	111	92.8	106	90.5	107	89.7
Hao	95	92.5	92	91.3	93	93.5	90	95.5	90	93.3	92	94.3
Average	112	91.6	117	90.9	108	92.4	107	93.7	106	90.4	110	92.0

CMP = Cow's milk powder diet

SMS = Soybean milk-substitute, formula 5410

SMS.HP = Soybean milk-substitute, high protein

CLO = Cod liver oil

Table 16. Protein Metabolism of Infants in Different Dietary Periods

	CMP	SMS	SMS CLO	CMP CLO	SMS.HP CLO
Average age of infants at start of dietary period (month/day)	2/12	2/12	3/21	3/20	4/22
Average body wt. in the period (kg)	5.12	5.26	6.17	6.21	6.83
Calories from protein (%)	15.5	15.3	15.3	15.5	20.3
Average caloric intake (kcal/kg/day)	112.0	117.0	108.0	107.0	106.0
Nitrogen intake (mg/kg/day)	698.0	732.0	667.0	663.0	864.0
Nitrogen excretion (feces) (mg/kg/day)	67.0	167.0	125.0	49.0	154.0
Nitrogen excretion (urine) (mg/kg/day)	440.0	368.0	380.0	455.0	514.0
Nitrogen absorption (mg/kg/day)	631.0	565.0	542.0	614.0	710.0
Nitrogen retention (mg/kg/day)	193.0	196.0	162.0	159.0	196.0
Nitrogen absorption rate (%)	90.4	77.2	81.3	92.6	82.2
Nitrogen retention rate (%)	27.3	26.6	25.3	24.1	22.6

CMP = Cow's milk powder diet

SMS = Soybean milk-substitute, formula 5410

SMS.HP = Soybean milk-substitute, high protein

CLO - Cod liver oil

DISCUSSION

The task of the committee on protein-energy requirements of the IUNS is to establish standards of protein and energy requirements. These standards will serve as a basis for the appraisal of food supplies vs food requirements and for planning food production for the entire world. When one considers that the average person consumes 2-3 lb of food daily or 1/3 to 1/2 ton annually, the importance of protein and energy requirement standards is appreciated. China is a large country with nearly one billion mouths to feed; a difference of only 5% in protein or calorie requirements means many million of tons of food per year.

Dietary protein and energy are closely interrelated. If the calorie content of a diet is inadequate, the protein content will be used to supply energy. Consequently, protein deficiency is much more likely to occur if a diet is lacking in calories. Also, it is important to know the minimum protein calories required in the human diet, and what kind of protein should be provided. Most people in China do not receive the protein-rich foods that characterize diets in the industrialized countries in Europe and North America. Study is necessary to determine the relative amounts of dietary protein and energy required by the Chinese population.

Few foods contain large amounts of protein, although it is distributed widely in nature. Animal foods have high quality protein and an abundant quantity. However, some plant foods also are significant protein sources. Soybean protein is complete and is similar to most animal proteins; the protein content of soybeans is high. Grain products contain less protein but because they are consumed in large amounts, contribute a significant proportion of protein to the traditional Chinese diet. Grains do not contain adequate amounts of lysine, but legumes are usually high in lysine content. A combination of these two foods, therefore, provides an amino acid pattern similar to the amino acid scoring pattern. Total protein in the Chinese diet is composed of 60-70% cereal protein, and approximately 11% of animal and legume proteins (24). From a nutritional standpoint, it is necessary to increase the amount of animal and soy protein. So animal husbandry, fish, management, and increased soybean production should be encouraged. The traditional Chinese diet that is high in plant protein and crude fiber may be beneficial in preventing coronary heart disease; however, the questions on the absorption and utilization of protein and energy remain to be answered.

Studies at Purdue University Agricultural Experiment Station have resulted in a special strain of corn, Zea Mays, which carries the gene and designated opaque-2. In opaque-2, the incomplete protein zein, as found in regular corn, is reduced to 1/2 the usual concentration, while the complete protein gluten is doubled. Both lysine and tryptophan are increased approximately 50%, and there is a better balance between leucine and isoleucine. The opaque-2 gene is now being introduced into the germ plasm of corn in China in an attempt to improve the quality of protein in man's diet and in the feed of farm animals. Our experiments showed that it also is effective in the prevention of pellegra (25). Active research is underway in China to develop and produce strains of rice, wheat, sorghum, and other grains that have increased protein content.

We have studied the effect of meal frequency in relationtothe health of the student in a middle school (13). The results showed that four meals a day are better than three meals for both better health and learning ability. We have yet to ascertain the number of meals/day that is most beneficical for adults and children. Each meal should include a balanced essential amino acid

mixture. The distribution of energy and protein intake in each meal must be studied also.

The Recommended Dietary Allowance of protein for infants is 3.5 g/kg body weight. This protein was derived primarily from cow's milk. If the protein is derived from human milk or has been treated to render it more digestible, the allowance may be in the range of 2-3 g/kg. There should be no question that human milk is the desirable source of nutrients for young infants even though it may not provide the levels recommended for all nutrients. It has been found from infant metabolic studies with a soybean milk substitute that the average protein intake was 4.5 g/kg/day and furnished 15% of the total calories and gave a satisfactory nitrogen retention of about 190 mg N/kg/day. Experiments with graded vegetable protein intake of 5-6 g/kg/day (soybean and corn) supplying 17-18% of the total calories should provide satisfactory nitrogen retention of 70-130 g/kg/day.

Distribution of the food supply according to age and physical activity is a reasonable method which not only prevents hunger, but also satisfies the protein and energy requirements.

REFERENCES

1. Chen Xue-Cun: Recommended Dietary Allowances. Chinese Med. J. 51:38. 1965 (in Chinese).

2. Institute of Health, Chinese Academy of Medical Sciences: Food Table, p. 368, 1977 (in Chinese).

3. Hsien Wu: Calorie requirements. Texas Rep. Biol. Med. 9: 854, 1951.

4. Hi Hui and Chuang Chien: The energy expenditure of college students. Acta Nutrimenta Sinica 2:32, 1957.

5. Liu Guang-Yuand et al.: Energy expenditure in iron and steel work. Ibid. 2: 221, 1957.

6. Yu Yong-Zhong et al.: Energy expenditure in metallurgical industry. Chinese J. Pub. Hlth. 11:70, 1965 (in Chinese).

7. Yu Yong-Zhong et al.: Energy expenditure in coal mining. Weisheng Yanjiu 8:86, 1979 (in Chinese).

8. Chen Zue-cun: Nutrition and work in heat. Chinese. J. Publ. Hlth. 5: 1289, 1958 (in Chinese).

9. Chen Xue-Cun et al.: Nutrition survey of building workers. Ibid. 3:23, 1956.

10. Chen Xue-Cun: Nutrition and work in high temperature environment. Zhongji Yikan 7:460, 1957 (in Chinese).

11. Passmore, R. and Durnin, J.V.G.A.: Human energy expenditure. Physiol. Rev. 35: 30, 1955.

12. Yu Xi-Xuang et al.: Energy expenditure of peasants. Remin Baojian 2:148, 1960 (in Chinese).

13. Chen Xue-Cun et al.: Preliminary report on the study of four meals in the senior middle school. Chinese J. Publ. Hlth. 2:223, 1955 (in Chinese).

14. Chen Xue-Cun et al.: Studies on the nutritional scrotal dermatitis. Chinese Med. J. 44:779, 1958 (in Chinese).

15. Chang Ying et al.: Studies on milk substitutes. VIII. The digestibility and energy utilization of soybean milk substitute in infants. Preceedings of the 6th Chinese Pediatrics Convention, 1964.

16. Wang Chen-Fa: Studies on the protein requirement of adults. Proceedings of the Annual Meeting of the Peking Association of Physiological Sciences, p. 191, 1961 (in Chinese).

17. Kung Lan-Cheng et al.: A nutrition survey of some workers in a mine in Kwangtung Province. Acta Nutrimenta Sinica 3:76, 1958.

18. Liu Dong-Sheng et al.: Effects of varied protein intake on growth and nitrogen metabolism. Proceedings of the Second Nutrition Convention. p. 28, 1979 (in Chinese).

19. Liu Sheng-Chie et al.: Studies on a milk substitute. VI. Absorption and retention of nitrogen, calcium and phosphorus of soybean milk substitute by infants. Proceedings of the Annual Meeting of the Peking Association of Physiolgoical Sciences, p. 186, 1961 (in Chinese).

20. Scheinberg, I.H. (ed.): Infant Metabolism. MacMillan, London, p. 127, 1956.

21. Chou Chi-Yuang: Studies of soybean milk substitutes in recent 40 years. Chinese J. Pub Hlth. 1:132, 1954 (in Chinese).

22. Zheng Su-Fang: Dietary survey of the children in a county kindergarten. Proceedings of the Second Nutrition Convention, p. 36, 1979.

23. Liu Sheng-Chie et al.: Indication for evaluating protein nutrition of children. Ibid, p.27, 1979.

24. Liu Dong-Sheng: Nutrition and Food. Shi Ping Ke Ji 2:1, 1980 (in Chinese).

25. Chen Xue-Cun: Pellagra prevention. Chinese Med. J. 93:785, 1980.

NUTRITION RESEARCH, Vol. 1, pp. 489-497, 1981
0271-5317/81/050489-09$02.00/0 Printed in the USA.
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DEPRESSED TRANSFORMATION RESPONSE BY SPLENIC LYMPHOCYTES FROM VITAMIN A-DEFICIENT RATS

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ABSTRACT

Concanavalin A (Con A)-stimulated lymphocytes isolated from the spleen of vitamin A-deficient rats showed decreased [^{3}H]thymidine incorporation compared to pair-fed and ad libitum-fed controls. This decrease was evident over a range of Con A concentrations (1-7 µg/ml), and the mitogen concentration which elicited maximum response was the same (2 µg/ml) for all three dietary groups. A three-day *in vivo* repletion with retinyl acetate led to recovery of normal or greater than normal [^{3}H]thymidine incorporation at all concentrations of Con A. At time intervals when DNA synthesis was significantly increased by Con A (48, 72 and 96 hours after stimulation) the [^{3}H]thymidine incorporation by vitamin A-deficient lymphocytes was always lower than that of pair-fed and ad libitum-fed controls. Decreased splenocyte blastogenesis was also demonstrated for the B-cell mitogen *E. coli* lipopolysaccharide, which was not completely accounted for by reduced numbers of B-cells in the spleen of vitamin A-deficient rats.

KEY WORDS: Vitamin A, Immunity, Transformation, Vitamin A-deficient

INTRODUCTION

In addition to the well known effect of vitamin A in the visual process, a number of physiological and biochemical lesions are associated with vitamin A deficiency. Vitamin A is necessary for proper development of reproductive organs (1), bone tissue (2), and mucus-secreting epithelia (3).

Recently there has been increased interest in the role of vitamin A as an immunoregulatory agent. A number of clinical studies have associated reduced plasma vitamin A levels with an increased incidence of a variety of infections (4), although PCM and other nutrient deficiencies were probably present in the populations studied. Cell-mediated immune function as measured by skin test reactivity (5), reduced peripheral T-cell levels (6), and PHA responsiveness (6) has been reported to be depressed in vitamin A-deficient children.

Vitamin A-deficient animals are more susceptible to viral (7), bacterial (8) and parasitic (9) challenge. More attention has focused on the stimulatory effect of vitamin A on immune function. High doses of vitamin A diet enhance

the resistance of laboratory animals to infection with Pseudomonas (10), Listeria (10,11), Candida (10) or E. coli (12). The vitamin appears to act as an adjuvant in stimulating antibody response to a variety of antigens (13-15), increasing the activity of the mononuclear phagocytic system (11), and accelerating graft rejection across minor histo-compatibility barriers (16). Histological changes consisting of hypercellularity, enlargement of the regional lymph nodes, and increased cellular traffic following injection of vitamin A alcohol are consistent with the observed immunologic changes.

We reported that vitamin A deficiency in rats leads to decreased numbers of circulating lymphocytes and reduced in vitro splenic lymphocyte response to the mitogens Concanavalin A (Con A), phytohemagglutinin (PHA), and E. coli lipo-polysaccharide (LPS) (17). The defects were shown to be independent of concomitant protein-calorie malnutrition and were reversed by a three-day in vivo repletion with retinyl acetate. Further experiments were necessary to determine if the depressed transformation response was due to alterations in the mitogen dose response curve or to a shift in the time of maximal stimulation. The question also arose whether the loss of LPS function was due to a decreased number of B-cells or to the loss of cell function. We report here that [^{3}H]thymidine uptake by splenic lymphocytes is reduced at all concentrations of the mitogen Con A and at all times during the course of the mitogenic response. Furthermore, reduced response to the B-cell mitogen LPS could not be accounted for by a simple reduction in the number of splenic B-cells.

MATERIALS AND METHODS

Animals and Diet

Male, weanling Lewis rats (Charles River Breeding Laboratories Inc., Wilmington, MA) were maintained as previously described (17) to establish vitamin A-deficient (DEF), pair-fed control (PF), and ad libitum-fed control (AL) rats. Animals in the DEF group were fed a commercial vitamin A-free rat chow (Teklad Inc., Madison, WI) while the AL and PF controls received the same diet supplemented with 500 µg of vitamin A (all trans retinyl acetate, Eastman Organic Chemicals, Rochester, NY). Rats were assumed to be vitamin A-deficient when they reached a weight gain plateau (18). Deficiency was later confirmed by measuring serum vitamin A concentration (19). As each vitamin A-deficient rat reached weight plateau, it and its PF and AL controls were killed and mitogen transformation studies conducted as described below.

In a second study, rats were fed the vitamin A-free diet until they achieved weight plateau or weight loss. Once deficient, they were paired; one rat of each pair was supplemented orally with 580 µg of retinyl acetate per day, while the other rat of the pair remained on the vitamin A-free diet. Rats were sacrificed after three days of supplementation.

Lymphocyte Transformation

We obtained blood samples by cardiac puncture with the animals under light ether anesthesia and then decapitated the rats. We placed the spleen in cold RPMI-1640 (M/A Bio-products, Walkersville, MD) and achieved a crude lymphocyte suspension by teasing the spleen followed by centrifugation and washing with RPMI. The cells were suspended in a medium consisting of 90% Eagle's Modified Suspension Medium (MEM), fetal bovine serum (10%; containing < 0.5 µg retinol/ml), penicillin and streptomycin (final concentration: 50 units and 50 µg/ml), and L-glutamine (final concentration: 2 mM), then counted on a Coulter Counter. In experiments where E. coli lipo-polysaccharide (LPS) was the mitogen, 5 x 10^{-5} M β-mercaptoethanol (β-ME) was included in the culture medium. Viability was determined by the trypan blue exclusion method.

Microwell cultures were set up and harvested as previously described (17). The Con A dose response and time course experiments, using 4 x 10^5 lymphocytes/well, were labeled with 1 µCi of [^{3}H]thymidine (40-60 Ci/mmol; New England

Nuclear, Boston, MA). For the mitogen dose response experiments, the amount of Con A was varied (0-7 µg/ml) while the pulse period was held at 48-72 hours. For the time course experiment, Con A concentration was held constant at 2 µg/ml while pulse time was varied (0-24, 24-48, 48-72, 72-96 hours). LPS (Sigma Chemical Co., St. Louis, MO) was made up to 100 µg/ml in sterile water, boiled for 2 hours and restored to original volume. Aliquots were frozen and used at final culture concentrations of 10-20 µg/ml. LPS cultures were harvested at 72 hours following a 24-hour pulse with [^{3}H]thymidine. All cultures were incubated in a high humidity, 5% CO_2 atmosphere at 37°C and harvested on glass fiber strips by an automatic sample harvester. [^{3}H]thymidine incorporation was determined by liquid scintillation counting and the results expressed as cpm. Values are given as mean ± SEM. Statistical differences were determined by Student's t-test.

B-cell Enriched Spleen Preparations

Splenocytes were resuspended in MEM and incubated at 37°C on a nylon wool column for 60 minutes (20). T-cells and null lymphocytes were eluted by washing the column with RPMI. Adherent cells (approximately 85% B-cells) were recovered by compressing the nylon wool with simultaneous rinses of RPMI. The number of B-cells in the various preparations was determined by previously described immunofluorescent techniques (17).

RESULTS

Con A Dose Response

The reponse of splenic lymphocytes to Con A was tested over a range of mitogen concentrations. The results confirmed earlier reports (17) of a depressed transformation response of splenocytes from vitamin A-deficient animals. The DEF group incorporated significantly less ($P < 0.01$) [^{3}H]thymidine into Con A-stimulated cells at 72 hours than the PF or AL groups (Fig. 1). This was true for all doses (1-7 µg/ml) of Con A tested. For other time intervals, the DEF-stimulated cultures were significantly lower than the PF controls.

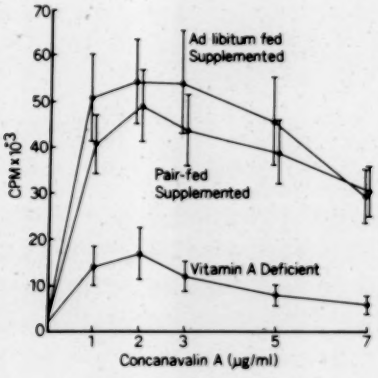

FIG. 1
Con A Dose Response
Splenic lymphocytes were isolated from vitamin A-deficient, pair-fed vitamin A-supplemented, and ad libitum-fed vitamin A-supplemented Lewis rats. Each vertical bar represents the mean ± SEM for 10 rats.

The same dose range of Con A (1-5 µg/ml) gave the maximum transformation response for all three groups, suggesting that the decreased response was not due to a change in sensitivity to the mitogen. DEF unstimulated cultures (Con A = 0 µg/ml) did not incorporate less thymidine than PF cultures, so the depression demonstrated in the stimulated DEF cultures was not due to a general malfunction of the lymphocytes.

The PF group, although malnourished, had a serum vitamin A level (47 ± 3 µg/dl) similar to the AL group (54 ± 1 µg/dl), while the DEF group had a very low serum vitamin A concentration (8 ± 2 µg/dl). The protein-calorie malnutrition of the PF rats, and DEF rats to which they were pair-fed, was evident in the average weight at the time of sacrifice; the PF group (283 ± 9 g) was similar to the DEF (287 ± 6 g) and both were significantly lighter than the AL group (318 ± g).

In the second study, the serum vitamin A concentration of deficient rats repleted with retinyl acetate was 41 ± 3 µg/100 ml, while the level of the vitamin A-deficient rats to which they were paired was 5 ± 1 µg/100 ml. The Con A response of the repleted group was significantly greater ($P < 0.05$) than the vitamin A-deficient group at all concentrations of the mitogen (1-7 µg/ml) (Fig. 2). Vitamin A status did not affect [^{3}H]thymidine uptake by unstimulated cultures; the value for the repleted group was 1232 ± 161, while the value for the DEF rats was 1900 ± 415.

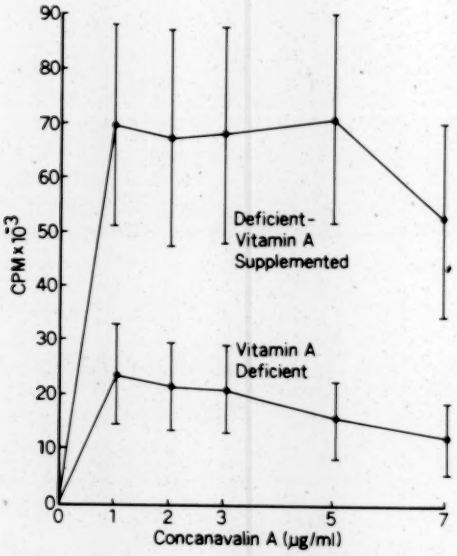

FIG. 2
Con A Dose Response (Vitamin A-repleted Animals)
Animals were treated as described under Animals and Diet, second study. Each vertical bar represents the mean ± SEM for 8 rats.

Effect of Varying Incubation Time on Transformation Response

Our results, and those previously published, document an effect of vitamin A-deficiency on DNA synthesis (as measured by [^{3}H]thymidine incorporation) over the 72 hours following mitogen stimulation. Zile et al. (21) speculated that

vitamin A deficiency slows the cell cycle; they reported a 23% increase in the length of the DNA synthesis phase of their intestinal, epithelial crypt cell model. To test whether their hypothesis applied to the decreases we observed in splenocyte transformation, we measured [^{3}H]thymidine uptake over other time time periods (Fig. 3)

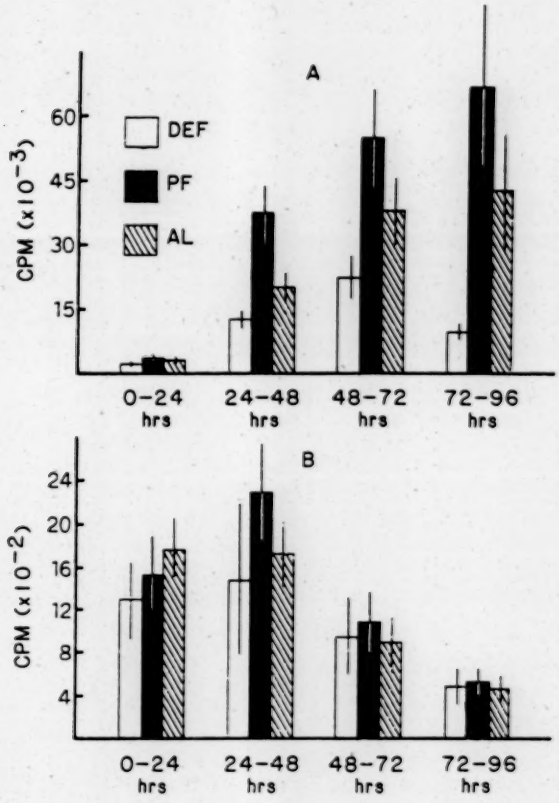

FIG. 3

Splenic Lymphocyte Transformation: Con A Time Course
The bars represent mean counts per minute of [^{3}H]thymidine incorporated into splenic lymphocytes of vitamin A-deficient (DEF), pair-fed control (PF), and ad libitum-fed control (AL) rats. Values are expressed as mean + SEM (n = 7). A: Cultures were stimulated with Con A (2 µg/ml). B: Unstimulated cultures.

At 24 hours, we saw no stimulation in cultures from any of the three dietary groups. After 48 hours, Con A-stimulated cells from the DEF groups incorporated significantly less radioactivity than those from PF or AL groups. There was no evidence that cultures from deficient animals were manifesting a

peak response earlier than the usual 72-hour harvest time. However, at 96 hours there was a decrease in [^{3}H]thymidine incorporation in the stimulated DEF cultures, but not in the lymphocytes from AL or PF animals. No differences were observed in thymidine uptake by unstimulated cells, which indicates that differences in lymphocyte viability do not account for the depression in lymphocyte transformation in splenocytes from DEF rats.

Variations in Splenic Cell Populations in DEF Animals

We chose two approaches to determine if the depressed LPS response observed in previous experiments (17) was due to a decreased number of B-cells in the DEF spleen or to a loss of cell function. We first determined the percentage of B-cells in splenic preparations and expressed the transformation results in LPS-stimulated cultures as cpm per 2 x 10^5 cells and cpm per 2 x 10^5 B-cells. Although the number of B-cells was reduced in preparations from DEF rats, it did not completely account for the reduction in LPS transformation response. Alternatively, cultures were set up with equal numbers of enriched B-cell preparations (see Materials and Methods) containing 70-85% B-cells and stimulated with LPS in the usual manner. Vitamin A-deficient rats demonstrated less LPS-stimulated [^{3}H]thymidine incorporation than either dietary control group. Furthermore, results from our experiment were similar to those obtained when the mathematical adjustment was made for B-cell content.

TABLE 1
LPS Transformation Response

Preparation	Vitamin A Def. (DEF)	Pair-fed Control (PF)	Ad lib.-fed control (AL)
Crude Spleen:			
Unstimulated (cpm)	4357 ± 570[1]	7652 ± 1160[3]	5677 ± 682[2]
LPS-stimulated (cpm)	7280 ± 1154	17927 ± 3067[3]	14001 ± 1489[3]
B-cells (%)	33 ± 2	40 ± 2[3]	39 ± 3[2]
Stimulated (cpm) ÷ % B-cells[4]	23920 ± 4482	45961 ± 6479[2]	41704 ± 4396[2]
Enriched B-Cell:			
LPS-stimulated (cpm)	22593 ± 3428	42585 ± 8592[3]	46879 ± 4265[3]

[1] All values expressed as mean + SEM (n = 7-10).
[2] Significantly different from DEF at $P < 0.05$ (Student's t-test).
[3] Significantly different from DEF at $P < 0.01$ (Student's t-test).
[4] Calculated by dividing individual experimental results by the percentage of B-cells in that preparation.

DISCUSSION

The depressed response to the mitogen Con A seen in splenic lymphocytes from DEF rats suggests that impaired cell-mediated immune function might be one factor responsible for the increased susceptibility to infection seen in vita- demonstrate the same defects indicates that the impaired lymphocyte transformation response and depressed WBC number (17) were not due to the accompanying protein-calorie malnutrition. The impairment in proliferation is not permanent, as it can be quickly reversed by vitamin A administration.

The mechanism by which vitamin A deficiency influences the function and circulation of lymphocytes is not understood. Although general protein-calorie malnutrition has been shown to depress serum factor thymic activity, specific vitamin A deficiency has been shown to have no effect in an animal model (22). Polyisoprenoid compounds (23,24), including retinol (25), have been implicated as cofactors in the synthesis of certain membrane glycoproteins. Impaired synthesis of glycoproteins has been demonstrated in the cornea (26), small intestine (3), and epithelial tissues (27) of vitamin A-deficient rats. Con A, a plant lectin known to stimulate T-cells, must bind to the lymphocyte surface glycoproteins to inititate transformation (28). Vitamin A deficiency could limit proper synthesis of the surface glycoproteins functioning as mitogen receptors on the lymphocyte, thereby interfering with the response to Con A.

An alternative hypothesis is that depressed thymidine incorporation could be due to loss or redirection of a mitogen responsive T-cell subpopulation. A subpopulation of T-cells has been characterized as circulating, cortisone-resistant, and mitogen responsive (29,30). Maturation of lymphocytes was shown to involve changes in glycosylation (30,31), and Woodruff and Gesner (32,33) demonstrated that interference with the glycoprotein surface alters the lymphocyte distribution. The depression seen in the WBC (17) supports the concept of a circulation defect, since greater than 60% of the rat's circulating lymphocytes are T-cells (34).

Further work is needed to determine changes in membrane glycoproteins and mitogen-binding capacity in lymphocytes from vitamin A-deficient rats. Preliminary experiments have indicated that labeled mesenteric lymphocytes have a different traffic pattern than cells from PF or AL controls (35).

ACKNOWLEDGEMENTS

This work was supported in part by NIH Grant RR-88. We wish to thank Henry Frechter for his contribution to this study.

REFERENCES

1. De LUCA, L.M. Vitamin A. In: The Fat Soluble Vitamins. H.F. DeLuca (ed). Plenum Press, New York, 1978.

2. MOORE, T. Vitamin A. Elsevier Publishing Co., New York, 1957, pp. 14-316.

3. De LUCA, L.M., SCHUMACHER, M. and WOLF, G. Biosynthesis of a fucose-containing glycopeptide from rat small intestine in normal and vitamin A deficient conditions. J. Biol. Chem., 245: 4551-4558, 1970.

4. GROSS, R.L. and NEWBERNE, P.M. Role of nutrition in immunologic function. Physiol. Rev., 60: 188-302, 1980.

5. JAYAKAKSHMI, V.T. and GOPALAN, C. Nutrition and tuberculosis. I. An epidemiologic study. Ind. J. Med. Res., 46: 87-92, 1958.

6. BHASKARAN, C. and REDDY, V. Cell-mediated immunity in iron- and vitamin-deficient children. Br. Med. J., 3: 522, 1975.

7. BANG, B.G., FOARD, M.A. and BANG, F.B. The effect of vitamin A deficiency and Newcastle disease on lymphoid cell systems in chickens. Proc. Soc. Exp. Biol. Med., 143: 1140-1146, 1973.

8. SOLOTOROVSKY, M., SQUIBB, R.L., WOGAN, G.N., SIEGEL, H. and GALA, R. The effect of dietary fat and vitamin A on avian tuberculosis in chicks. Am. Rev. Respir. Dis., 84: 226-235, 1961.

9. DARIP, M.D., SIRISINHA, S. and LAMB, A.J. Effect of vitamin A deficiency on susceptibility of rats to angiostrongylus cantonensis. Proc. Soc. Exp. Biol. Med., 161: 600-604, 1979.

10. COHEN, B.E. and ELIN, R.J. Vitamin A induced non-specific resistance to infection. J. Infect. Dis., 129: 597-600, 1974.

11. HOF, H. and EMERLING, P. Stimulation of cell-mediated resistance in mice to infection with histeria monocytogenes by vitamin A. Ann. Immunol. (Paris), 130C: 587-594, 1979.

12. TENGERDY, R.P. and BROWN, J.C. Effect of vitamin E and A on humoral immunity and phagocytosis in E. coli infected chickens. Poult. Sci., 59: 957-963, 1977.

13. JURIN, M. and TANNOCK, I.F. Influence of vitamin A on immunological response. Immunology, 23: 283-287, 1972.

14. COHEN, B.E. and COHEN, I.K. Vitamin A: adjuvant and steroid antagonist in the immune response. J. Immunol., 111: 1376-1380, 1973.

15. FALCHUK, K.R., WALKER, W.A., PERROTTO, J.L. and ISSELBACHER, K.J. Effect of vitamin A on the systemic and local antibody responses to intragastrically administered bovine serum albumin. Infect. Immun., 17: 361-365, 1977.

16. FLOERSHEIM, G.L. and BOLLAG, W. Accelerated rejection of skin homografts by vitamin A acid. Transplantation (Baltimore), 15: 564-567, 1972.

17. NAUSS, K.M., MARK, D.A. and SUSKIND, R.M. The effect of vitamin A deficiency on the in vitro cellular immune response of rats. J. Nutr., 109: 1815-1823, 1979.

18. COREY, J. and HAYES, K.C. Cerebrospinal fluid pressure, growth and hematology in relation to retinol status of the rat in acute vitamin A deficiency. Biochim. Biophys. Acta, 265: 417-441, 1972.

19. HANSEN, L.G. and WARWICK, W.J. A fluorometric micromethod for serum vitamin A. Am. J. Clin. Pathol., 50: 525-529, 1968.

20. TRIZIO, D. and CUDKOWICZ, G. Separation of T and B lymphocytes by nylon wool columns: evaluation of functional assays in vitro. J. Immunol., 113: 1093-1097, 1974.

21. ZILE, M., BUNGE, E.C. and DeLUCA, H.F. Effect of vitamin A deficiency on intestinal cell proliferation in the rat. J. Nutr., 107: 552-560, 1977.

22. CHANDRA, R.K., HERESI, G. and AU, B. Serum thymic factor activity in deficiencies of calories, zinc, vitamin A and pyridoxine. Clin. Exp. Immunol., 42: 332-335, 1980.

23. LENNARZ, W.J. and SCHER, M.G. Metabolism and function of polyisoprenol sugar intermediates in membrane-associated reactions. Biochim. Biophys. Acta, 265: 417-441, 1972.

24. WAECHTER, C.J. and LENNARZ, W.J. The role of polyprenol-linked sugars in glycoprotein synthesis. Annu. Rev. Biochem., 45: 95-108, 1976.

25. ROSSO, G.C., De LUCA, L., WARREN, C.D. and WOLF, G. Enzymatic synthesis of mannosyl retinyl phosphate from retinyl phosphate and guanosine diphosphate mannose. J. Lipid Res., 16: 235-243, 1975.

26. KIM, Y.L. and WOLF, G. Vitamin A deficiency and the glycoproteins of rat corneal epithelium. J. Nutr., 104: 710-718, 1974.

27. BONANNI, F., LEVINSON, S.S., WOLF, G. and De LUCA, L. Glycoproteins from the hamster respiratory tract and their response to vitamin A. Biochim. Biophys. Acta, 297: 441-451, 1973.

28. SHARON, N. and LIS, H. Lectins: cell agglutinating and sugar-specific proteins. Science, 177: 949-959, 1972.

29. SCHLESINGER, M., SHLOMAI-KORZASH, Z. and ISRAEL, E. Antigenic differences between spleen-seeking and lymph node-seeking thymus cells. Eur. J. Immunol., 3: 335-339, 1973.

30. GOLDSCHNEIDER, I. Antigenic relationship between medullary thymocytes and a subpopulation of peripheral T-cells in the rat: description of a masked antigen. Cell. Immunol., 16: 269-284, 1975.

31. MISRA, D.N., LADOULIS, C.T. and GILL, I.J. Lymphocyte plasma membranes. VI. Plasma membrane glycoproteins of thymic and splenic lymphocytes from inbred rats. Biochim. Biophys. Acta, 506: 221-232, 1978.

32. WOODRUFF, J. and GESNER, B.M. The effect of neuraminidase on the fate of transfused lymphocytes. J. Exp. Med., 129: 551-567, 1969.

33. WOODRUFF, J. and GESNER, B.M. Lymphocytes: circulation altered by trypsin. Science, 161: 176-178, 1968.

34. BALCH, C.M. and FELDMAN, J.D. Thymus-dependent (T) lymphocytes in the rat. J. Immunol., 112: 79-86, 1974.

35. McDERMOTT, M.R., MARK, D.A., BEFUS, A.D., BALIGA, B.S., SUSKIND, R.M. and BIENENSTOCK, J. Defective intestinal lymphocyte localization associated with protein-calorie malnutrition and vitamin A malnutrition. Fed. Proc., 40(3): 1103, 1981.

NUTRITION RESEARCH, Vol. 1, pp. 499-507, 1981
0271-5317/81/050499-09$02.00/0 Printed in the USA.
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FEEDING BEHAVIOR OF THE CAT FED LABORATORY AND COMMERCIAL DIETS[1]

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ABSTRACT

Using a continuous recording computerized system, an experiment was conducted to examine the feeding and drinking patterns of the cat. Four diets; 1), dry commerical; 2), canned commercial; 3), casein based purified diet; and 4), amino acid based purified diet were fed to 8 adult (3-8 kg) cats during 8 consecutive 7-10 day periods. Mean daily dry matter intake was greater ($P < .01$) for the dry than the canned diets. Mean daily caloric intake was 71 kcal/kg BW. Drinking water intake was greater ($P < .01$) during feeding of diet 1. Total water intake (mls/g DM) was highest ($P < .01$) for diet 2, even though the cats drank no water. There were no differences in meal frequency among diets (16 meals/day). Mean meal size (g DM/meal) was 4.8 g. It is concluded that domestic cats eat many small meals throughout the 24 h period, showing no diurnal cycle. After only a brief adaptive period cats clearly adjusted their dietary intake to their maintenance requirement despite the variable caloric content of the 4 diets.
KEY WORDS: Food intake, cat, water intake.

INTRODUCTION

There are little available data on the diurnal feeding and drinking patterns of the cat. It has previously been suggested that cats are intermittent or occasional feeders (1, 2), eat frequently throughout the day (3, 4) or that they eat predominately during the dark (5) period. This paper describes the results of simultaneous measurement of food and water intake of adult cats fed laboratory and commercial diets. It also describes the methodology used to obtain such data.

MATERIALS AND METHODS

Animals. Eight specific pathogen-free, two to three-year-old cats (4 castrated males, 4 spayed females; 3-8 kg body weight) were used. They had previously been fed various purified and commercial diets throughout their life.

[1]Supported in part by NIH grant T32-AM07355-02 and AM13252.

Diets. Two commercial and two purified diets were fed to each cat. Diet 1, was a dry commercial expanded product (Crave[2], Kal Kan Foods, Vernon, CA); diet 2, was a high moisture commercial gourmet canned product (Seaside Supper[3], Kal Kan Foods, Vernon, CA); diet 3, was a casein based purified diet (Table 1); and diet 4, was an amino acid based purified diet (Table 1). The diets and deionized water were available ad libitum.

Equipment. The cats were housed in individual metabolism cages (0.66 x 0.66 x 0.76 m) (SEFCO, Los Angeles CA) modified for food intake measurement (Fig. 1). A 14.0 cm hole was cut in the side of the cage and fitted with a rubber gasket at the level of the rear platform, to facilitate feeding from the food intake system. The aluminum and plexi-glass feeder (Fig. 1) was attached to the side of the cage. The hole at the rear of the feeder was of equal diameter and concentric with the hole on the cage. The side opposite the entrance to the feeder was made of plexiglass, providing light inside and allowing the cat to see out. The floor of the feeder was made of stainless steel screen (1.3 cm openings) with a 10.8 cm hole to facilitate feeding. Cats ate standing on the rear cage platform, extending their heads through the cage wall opening and through the opening in the floor of the feeder. Below the feeder was a double bowl (Fig. 1) which rested on the balance. The bowl was made of two concentric aluminum pans. This, along with the screen, allowed for spillage during feeding to return to the bowl and thereby remain on the balance. The bowl was fixed by two screws to the balance plate. The balance (Mettler PL 300-02, Mettler Instrument Corporation, Princeton NJ) was connected to the electronic monitoring system (7). Water was kept in a reservoir, resting on the top of the cage. The water flowed by tubing to the nipple (Lixit Value Co., Napa CA). The cats were previously trained to drink from the nipple. Water intake was monitored by a volumeter system utilizing a solenoid, electrode feedback system (8) modified for use with cats.

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[2]Crave, Kal Kan Foods, Los Angeles, California.

Guaranteed Analysis: Crude Protein, Min. 32.5%; Crude Fat, Min. 8.0%; Crude Fiber, Max. 4.5%; Moisture, Min. 12.0%; Calcium, Min. 1.0%; Phosphorus, Min. 0.8%; Salt (NaCl), Max. 1.5%.

Ingredients: Chicken by-product meal, ground yellow corn, wheat millrun, soybean meal, corn gluten meal, digest of (poultry by-products, chicken parts and tuna), dried milk protein, animal fat preserved with BHA, tuna meal, iodized salt, dried yeast, artificial colors, vitamin A supplement, vitamin D_3 supplement, niacin, vitamin E supplement, thiamine (vitamin B_1), Riboflavin supplement (vitamin B_2), pyridoxine hydrochloride (vitamin B_6), vitamin B_{12} supplement, calcium pantothenate, biotin, choline chloride, cobalt carbonate.

[3]Seaside Supper, Kal Kan Foods, Los Angeles, California.

Guaranteed Analysis: Crude Protein, Min. 10.0%; Crude Fat, Min. 6.0%; Crude Fiber, Max. 1.5%; Moisture, Max. 78.0%; Ash, Max. 3.0%; vitamin E, 37.8 IU/kg; vitamin A, 6952 IU/kg: vitamin D_3, 277 IU/kg; choline, 836 mg/kg; thiamine, 1.3 mg/kg; biotin, .07 mg/kg.

Indegredients: Fish, water sufficient for processing, poultry by-products, meat by-products, ground corn, soy flour, vegetable gums, iodized salt, choline chloride, vitamin A, D_3, and E supplements. magnesium oxide, iron sulfate, thiamine (B_1), manganous sulfate, zinc sulfate, pyridoxine hydrochloride (B_6), copper sulfate, folic acid.

Table 1

Experimental diets

	Diet 3 %	Diet 4 %
Casein	26.5	-
Amino Acid Mixture	1.9[1]	24.0[2]
Beef Tallow	25.0	25.0
Sucrose	17.1	18.7
Starch	21.5	24.1
Mineral Mixture[a]	5.0	5.0
Vitamin Mixture[b]	1.0	1.0
Sodium Acetate	0.5	1.6
Choline Chloride	0.5	0.5
	100.0	100.0
Solka Floc	1.0	1.0

[1]This mixture contained (%) ArgHCl, 60.0; cys-cys, 26.9; hist, 0.8; met, 2.7; thr, 9.6.

[2]Morris and Rogers(6).

[a]Mineral mix (%): $CaHPO_4$, 39.0, K_2HPO_4, 9.0, $CaCO_3$, 11.0; $MgSO_4$, 4.5. KCl, 10.0; $KHCO_3$, 10.0; $NaHCO_3$, 14.0; $MnSO_4 \cdot H_2O$, 0.38; $ZnSO_4 \cdot 7H_2O$, 0.44; $CuSO_4 \cdot 5H_2O$, 0.08; $FeC_6H_5O_7 \cdot H_2O$, 1.0; pentacalcium orthoperiodate (29% I), 0.015; $SnCl_2 2H_2O$, 0.01; Na_2SeO_3, 0.003; $(NH_4)_2MO_7O_4 \cdot 4H_2O$ 0.004; $CrCl_3 \cdot 6H_2O$, 0.026; $NiCl_2 \cdot 6H_2O$, 0.030; NaF, 0.014; $NH_4VO_3 \cdot 4H_2O$, 0.0025; NaCl .487.

[b]Vitamins per kg diet; retinylacetate, 20,000 IU; cholecalciferol, 2,000 IU; D,L-α-tocopheryl acetate, 160 IU; menaquinone, 15 mg; thiamin HCl, 25 mg; riboflavin, 10 mg; pyridoxine, 10 mg; nicotinic acid, 100 mg; D-Ca pantothenate, 20 mg; myo-inositol, 200 mg; folic acid, 10 mg; D-biotin, 1 mg; cyanocobalamin, 50 µg; ascorbic acid, 200 mg; taurine 750 mg; choline dihydrogen citrate 7.31 g.

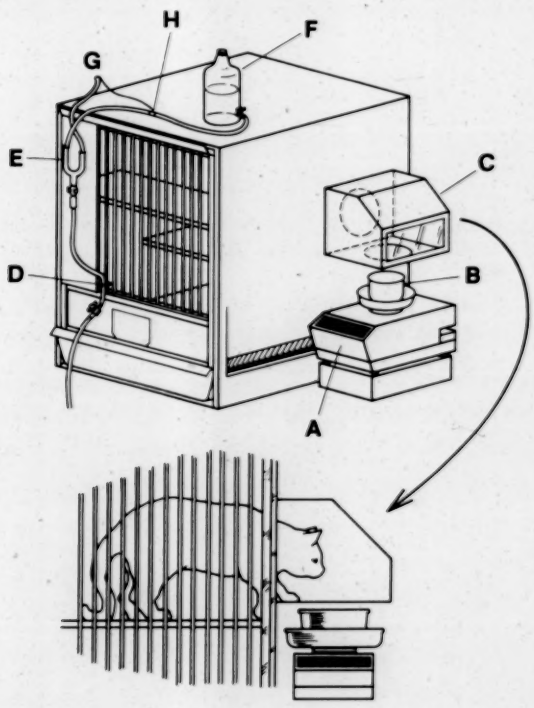

FIG. 1

Food and water intake apparatus. A. balance; B. concentric food bowl; C. feeder; D. nipple; E. U-tube; F. Water reservior; G. electronic connection; H. Solenoid.

Design. Eight cats were fed each of the four diets, during eight consecutive 7-10 day periods, in a randomized design except that the cats were fed the two commercial diets or two purified diets in sequence to enhance acceptance. The room was maintained at 22°C and 50% relative humidity. Lighting was maintained on a 12-hour light-dark cycle. The lights came on at 12 P.M.

Measurements. Food and water intake measurements were simultaneously and continuously recorded via the electronic monitoring system utilizing a RT-11 computer system (7) (Digital Equipment Corp. Maynard MA). The cats were fed at 12 P.M. daily, after the computer system was disengaged. Balance weights and water reservoir levels were recorded before and after refeeding. During this period (usually about 30 min.) the cats were prevented from eating, by a metal plate placed between the feeder and cage wall. The computer was then re-engaged to begin the next period. Since the balance was limited to ~350 g, cats fed diet 2 were also fed at 5 p.m., in order to maintain ad libitum feeding. During this period (5 min.) all cats were prevented from feeding. Samples of each diet were analysed for dry matter and gross energy content. The cats were weighed at weekly intervals.

Statistical Methods. Data were analysed statistically using one-way analysis of variance and the Student-Newman-Keuls test (9).

RESULTS AND DISCUSSION

The cats adapted rapidly to the food and water intake apparatus, after a short training period. One cat's behavioral pattern precluded measurement of food intake for the purified and amino acid diets as he continuously knocked the bowl off the balance. One cat failed to eat during one period and in two periods one balance and one watering device malfunctioned. These errors lowered the n value as shown in Table 3. Despite these problems the computerized system used in this study was an effective method for obtaining data on the feeding and drinking patterns of the cat.

Analyses of the diets are shown in Table 2. Dry matter, crude protein and energy content varied between diets. The cats mean food intake (g DM/day) for diets 1-4 are shown in Table 3. Mean daily food intake for the cats fed the dry commercial diet was greater ($P < .01$) than for the canned diet which is in agreement with other published work (10).

It has been reported that cats drink 6 times as much water when fed a dry than when fed a canned ration and that the drinking water to dry matter intake ratio was greater (10). Mean drinking water intake was greater ($P < .01$) when the cats were fed dry diet (commercial or laboratory) than canned food (Table 3). After 2 days none of the cats fed the canned diet drank any water. Drinking water intake was greater during periods when the dry commercial diet was fed than when feeding either of the purified diets (table 3). Mean drinking frequency (bouts/day) and mean bout size (ml/bout) for cats fed diets 1-4 is shown in Table 4. There were no significant differences in mean drinking frequency and drinking bout size between diets 1, 3 and 4, though as expected, each was greater ($P < .01$) than when the cats were fed the commercial canned diet. Total water intake from both drinking and % moisture contained in the food (mls/g DM intake) are shown in Table 3. Total water intake, was greater when cats were fed the canned ration, even though the water intake was entirely from the diet. This is in agreement with the findings of Thrall and Miller (12) who reported total water to dry matter intake ratios of 2.3:1 for cats fed dry diets and 3.3:1 fed meat diets. On the basis of total water intake Seefelt and Chapman (10) reported a greater ratio for those fed the canned ration. It has also been shown (13) that cats fed all meat diets could survive without drinking water. During feeding of the canned diet cats did not drink any water after the first or second day of the period. Some cats drank 10-40 mls of water these first days and then only 0-1 ml each day afterwards. The water consumed with the diet (78%) was presumably enough to maintain their water requirement and was greater ($P < .01$) than that consumed while fed the dry diets. It has been shown that water intake might be involved in the etiology of urolithiasis (14). It is clear based on these data and previous work (14, 15) why cats predisposed to urolithiasis should be fed a high moisture diet.

Mean daily meal frequency was similar for all diets as is shown in Table 4. Mean number of meals per day (16.0) was greater than reported by Mugford (3) or Kanarek (5). Mean meal size (g DM/meal) for cats fed diets 1-4 and mean meal frequency (meals/period) are shown in Table 4. Although the cats were fed at the beginning of the light cycle they ate many small meals, repeatedly throughout the light and dark periods. Representative patterns of feeding and drinking are shown in

Table 2

Diet Analysis

Diet	%DM	kcalGE/g	kcalGE/g DM	*kcalME/g DM	%Crude Protein
Crave	92.3	4.5	4.9	4.2	36.9
Seaside Supper	23.4	1.5	6.5	4.6	45.4
Purified Diet	95.5	5.4	5.7	5.2	29.9
Amino Acid Diet	95.7	5.0	5.3	5.2	24.0

*Metabolizable energy calculated using 4 kcal ME/g crude protein, 4 kcal ME/g NFE, 9 kcal ME/g crude fat. Estimated mean ME intake (kcal/kg BW) for diets 1-4 were: 59.0, 47.7, 66.9, 62.4.

**Estimated crude protein per gram dry matter. Estimated mean daily crude protein intake (g) for diets 1-4 were: 29.4, 26.1, 21.6, 16.9.

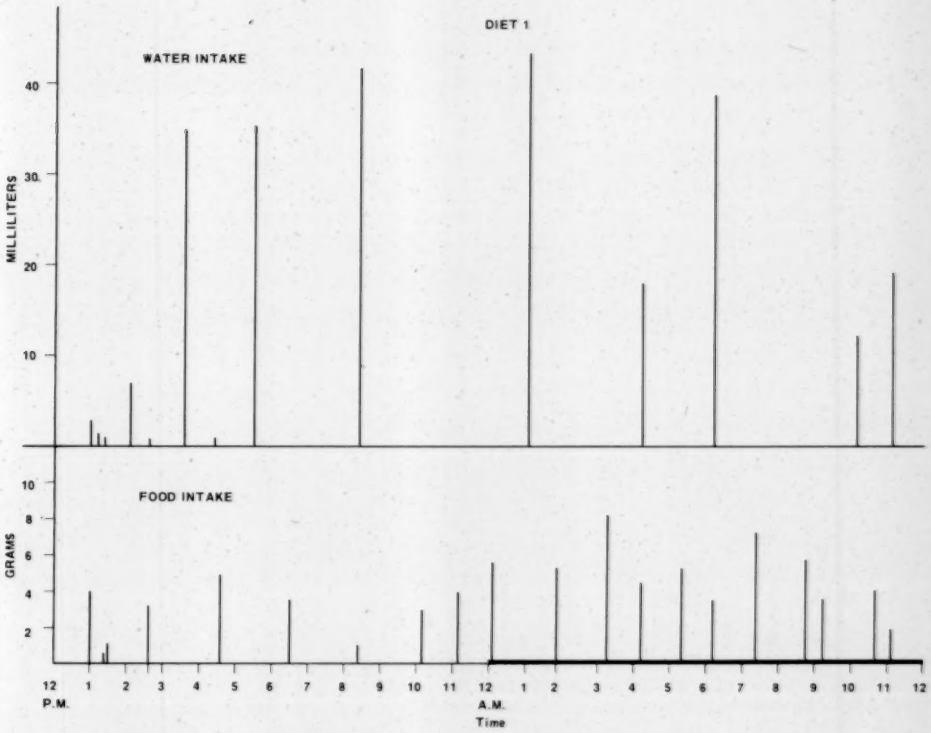

FIG. 2

Food and water intake patterns for cat fed diet 1.

Table 3

Food and water intake (Mean ± SE)

	Commercial		Purified	
Diet	Dry	Canned	Casein	Amino Acid
Food Intake				
g/day	86.3 ± 6.8[a]	256.1 ± 25.1[b]	75.6 ± 12.8[a]	73.7 ± 6.6[a]
gDM/day	79.7[a]	57.4[b]	72.2[a]	70.5[a]
kcal/kg BW	74.9 ± 7.9	73.1 ± 7.2	71.8 ± 5.5	65.8 ± 3.9
	n = 8	n = 8	n = 6	n = 5
Water Intake				
ml/day	147.3 ± 15.7[a,e]	0*[b]	79.4 ± 10.6[c]	98.1 ± 25.8[a,c,f]
ml/g DM	1.8	-	1.1	1.4
TW[+]ml/g DM	1.9[a]	3.5[b]	1.1[a]	1.4[a]
	n = 8	n = 8	n = 6	n = 5

* First day most cats drank some water, although intake dropped to zero by second or third day of period.

[+] Total water (TW) = drinking + moisture in food.

a, b, c values in the same row with different superscirpts differ ($P < .01$);
e, f values in the same row with different superscripts differ ($P < .05$).

Table 4

Feeding and drinking patterns

	Commercial		Purified	
Diet	Dry	Canned	Casein	Amino Acid
Feeding				
no. meals/day	15.7 ± 1.4	16.6 ± 1.8	16.8 ± 2.3	17.4 ± 3.5
meals/light	8.6 ± .9	10.7 ± 1.2	10.4 ± 1.2	10.6 ± 2.2
meals/dark	7.1 ± .7	5.9 ± .8	6.3 ± 1.3	6.7 ± 1.5
% Light/dark	55/45 ± 1.9[c]	65/35 ± 2.7[d]	64/36 ± 3.4[d]	62/38 ± 3.5[d]
g/meal	5.8 ± 7[a]	18.6 ± 4.5[b]	4.9 ± 1.0[a]	5.2 ± 1.2[a]
g DM/meal	5.3	4.2	4.7	5.0
	n = 8	n = 8	n = 6	n = 5
Drinking				
no. bouts/day	16 ± 1.3[a]	0[b]	12.5 ± 1.8[a]	12.4 ± 2.1[a]
mls/bout	10.5 ± 1.9[a]	0[b]	6.9 ± 1.1[a]	8.9 ± 2.2[a]
	n = 8	n = 8	n = 6	n = 5

a,b values in the same row with different superscripts differ ($P < .01$);
c,d values in the same row with different superscripts differ ($P < .05$).

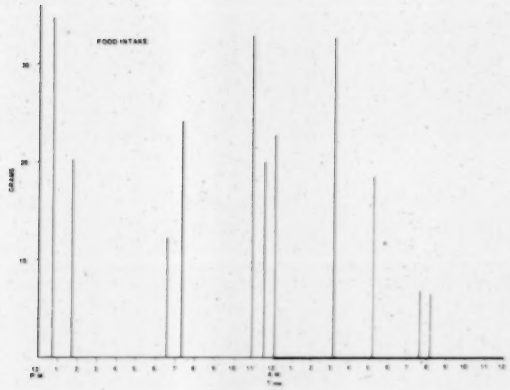

FIG. 3

Food intake patterns for cat fed diet 2.

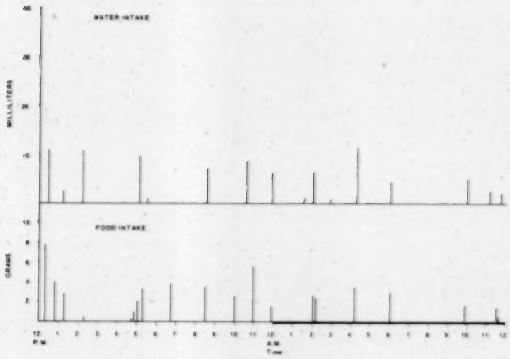

FIG. 4

Food and water intake patterns for cat fed diet 3.

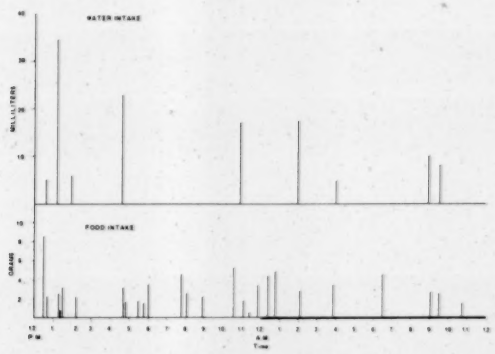

FIG. 5

Food and water intake patterns for cat fed diet 4.

figures 2-5. Although no significant correlations were found between meal size and post-meal intervals for all diets, significant correlations were found between meal size and pre-meal intervals for the canned ($r = .19$) and dry ($r = .32$) commercial diets. This agrees with data reported for the cat by Kanerek (5) though not with other work with the cat (16), rat (17), or other species (17-18), where no correlations have been shown. The number of meals eaten during the light period was greater ($P < .05$) than those eaten during the dark period for diets 2-4. Since the cats were fed during the light period further research needs to be conducted to study eating patterns in cats fed during the dark period. Mugford (3, 4) had previously reported that cats ate dry and canned diets throughout the 24-hour period, which is in agreement with this study; although Kanarek (5) had reported that cats ate predominantly in a noctural pattern. It appears that the eating pattern found here is similar to that of wild domestic cats eating rodents or other small animals (3). The cats in our study ate 23 kcal per meal, a single 20 g mouse would provide about 30 kcal. Mean meal size of 5.8 g for cats fed the dry commercial diet is in agreement with that found in earlier work (5). Mean caloric intake (kcal/kg BW/day) for diets 1-4 are shown in Table 3. Mean daily caloric intake (71 kcal/Kg BW) also agrees with other data obtained on the adult cat (11). From these results it appears that cats are able to regulate food intake via caloric consumption. There were no differences in daily caloric intake for cats fed diets ranging in caloric value from 4.9 to 6.5 kcal/g DM. This also suggests that the conclusions of previous work (5, 19) that cats don't regulate caloric intake when fed diets of various caloric densities, were possibly a result of high levels of the less palatable diluents, cellulose and kaolin, used. It would be interesting to determine caloric intake after dilution with more palatable substances.

ACKNOWLEDGEMENTS

The authors wish to express their appreciation to Roger Reidelberger and Dan Harrington for their technical assistance.

LITERATURE CITED

1. Scott, P. P. The special features of nutrition of cats with observations on wild felidae nutrition in the London Zoo. Supp. Zool. Soc. London. 21: 21-36, 1968.

2. Scott, P. P. Dietary requirements of the cat in relation to practical feeding problems. Paper Small Anim. Nutr. Workshop, Univ. Ill. Coll. Vet. Med. 1971.

3. Mugford, R. A. External influences on the feeding of carnivores. In: The Chemical Senses and Nutrition. II. (M. Kare and O. Maller, ed.) pp. 25-49. Academic Press, New York, 1977.

4. Mugford, R. A. and Thorne, C. Comparative studies of meal patterns in pet and laboratory housed dogs and cats. In: Nutrition of the Dog and Cat. (R. S. Anderson, ed.). pp. 3-14. Pergamon Press, New York, 1980.

5. Kanarek, R. B. Availability and caloric density of the diet as determinants of meal patterns in cats. Physiol. and Behav. 15: 611-618, 1975.

6. Morris, J. G. and Rogers, Q. R. Arginine: an essential amino acid in the cat. J. Nutr. 108: 1944-1953, 1978.

7. Leung, P. M. B., Rogers, Q. R., Stern, J. S., Mendel, V. E. An on-line computerized feeding, drinking and activity patterns analysis system. XI Annual Meeting Society for Neuroscience. Abstract. 1981.

8. Reidelberger, R. The role of postgastric mechanisms in the control of short term feeding behavior in the rat. Ph.D. Thesis. Univ. California, Davis, 1981.

9. Sokal, R. R. and Rohlf, F. J. Biometry. The Principles and Practices of Statistics in Biological Research. pp. 204-252. W. H. Freeman and Co., San Francisco, 1969.

10. Seefeldt, S. L. and Chapman, T. E. Body water contents and turnover in cats fed dry and canned rations. Am. J. Vet. Res. 40: 183-185, 1979.

11. National Research Council. Nutrient Requirements of Cats, National Academy of Sciences, Washington, D.C., 1978.

12. Thrall, B. E. and Miller, L. G. Water turnover in cats fed dry rations. Feline Pract. 6: 10-17, 1976.

13. Prentiss, P. G., Wolf, A. V. and Eddy, H. A. Hydropenia in cat and dog. Ability of the cat to meet its water requirements solely from a diet of fish or meat. Am. J. Physiol. 196: 625-632, 1959.

14. Barker J. and Povey R. C. The feline urolithiasis syndrome: a review and inquiry into the alleged role of dry cat foods in its aetiology. J. Small Anim. Pract. 14: 445-456, 1973.

15. Holme, D. W. Research into feline uroligical syndrome. Proc. Kal Kan Symposium for treatment of dog and cat diseases. Ohio State University. pp. 40-45, 1977.

16. Kaufman, L. W., Collier, G., Hill, W. L., and Collins, K. Mean cost and meal patterns in an uncaged domestic cat. Physiol. Behav. 25: 135-137, 1980.

17. DeCastro, J. M. Meal pattern correlations: Facts and antifacts. Physiol. Behav. 15, 13-15, 1975.

18. Chase, L. E., P. J. Wanganess and B. R. Baumgardt. Feeding behavior of steers fed a complete mixed ration. J. Dairy Sci. 59: 1923-1928, 1976.

19. Hirsch, E., Dubose, C. and Jacobs, H. L. Dietary control of food intake in cats. Physiol. Behav. 20: 287-295, 1978.

NUTRITION RESEARCH, Vol. 1, pp. 509-517, 1981
0271-5317/81/050509-09$02.00/0 Printed in the USA.
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CHANGES IN SERUM TRIGLYCERIDES, RETINOIC ACID, AND RETINOL ASSOCIATED WITH WITHDRAWAL OF RETINOIC ACID FROM THE DIET OF RATS

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ABSTRACT

Several experiments were performed to measure changes in serum triglycerides, retinoic acid, and retinol following removal of all-trans retinoic acid from the diet of rats previously fed 25 µg all-trans retinoic acid/g of diet for periods of 8-13 days. Inititally, rats had hypertriglyceridemia, but within 24-36 hours after withdrawal of retinoic acid, serum triglycerides returned to the level of control rats not fed retinoic acid. Serum retinoic acid also decreased after withdrawal, but did not return to the level of the control rats until 3 days afterwards. Serum retinol was depressed compared to the level of control rats until 72 hours after retinoic acid withdrawal. The fatty acid composition of the individual lipid fractions was shifted towards oleate and linoleate and away from palmitate and arachidonate after retinoic acid feeding. Following removal, restoration of a more normal pattern ensued.

KEY WORDS: retinoic acid, vitamin A, retinol, triglycerides, hyperlipidemia

INTRODUCTION

An elevation of serum triglycerides accompanies the administration of vitamin A at levels exceeding those normally ingested from dietary sources (1-3). Recently, clinical and experimental administration of vitamin A analogs (retinoids) has also been associated with hypertriglyceridemia (4-6). The mechanism by which this elevation occurs remains obscure, although various theories have been advanced (1, 5). Observations have also been made that administration of retinoic acid depresses the level of retinol in the blood of rat and man (3, 4, 7,8).

The current study was designed to measure changes in serum lipids, retinol and retinoic acid following removal of all-trans retinoic acid from the diet of rats administered this compound for eight days. These changes were monitored for up to 72 hours after the last administration of all-trans retinoic acid. Specific goals were to determine the time required for removal of dietary retinoic acid from serum, the time required for restoration of normal triglyceride levels and the possible relationship between retinol and retinoic acid serum levels in the retinoic acid-fed rat.

MATERIALS AND METHODS

Treatment of Animals

Male Sprague-Dawley rats (Harlan Industries, Inc., Cumberland, IN) weighing 135-160 g were housed individually in hanging wire cages in a controlled temperature environment (22°C) with a light cycle from 0600-1800 hours. The rats were fed an agar gel diet as described previously (3), composed of corn starch, sugar, casein, corn oil and adequate vitamins without vitamin A (Teklad, Madison, WI) and minerals (USP XVII), at the start of the dark cycle, with water offered ad libitum.

Blood samples were taken by cardiac puncture between 0930-1030, after the rats were anesthetized with ether. The blood was allowed to clot, centrifuged at 1500 x g for 30 min., and the serum was stored at -20° until analysis. For the second experiment, blood was taken, rats were guillotined, and their livers removed, washed with distilled water, patted dry, weighed and stored at -20° until analysis.

Extraction of Serum Retinol and Retinoic Acid for HPLC Analysis

All handling of vitamin A compounds was done in indirect light. The serum extraction technique used was a modification of the procedure of Ito et al. (9). The solvent extract was evaporated to dryness under nitrogen and its residue dissolved in 200 µl methanol for use in HPLC.

Samples of serum plus added retinol, retinoic acid and ^{14}C-all-trans retinoic acid dissolved in methanol, in the range to be assayed in serum, were processed through this same procedure to assess the adequacy of extraction. Similar standards of retinol and retinoic acid were chromatographed and their peak heights used as a measure of the linear response of the compounds.

High-Pressure Liquid Chromatography

All chemicals and solvents used were analytical reagent grade. Reversed-phase HPLC separation and analysis were done on a DuPont Instruments Zorbax ODS column (4.0 mm x 25 cm). The mobile phase contained methanol: 1.0% ammonium acetate (80:20) and was degassed and filtered through Whatman No 4 paper before it was pumped through the system isocratically at 1.1 ml/min. The peaks were recorded at the maximum sensitivity (0.005 AUFS). In this system retinol and retinoic acid eluted within 70 minutes. A Tracor Instruments Model 970A variable wave length detector set at 350 nm was used for monitoring the separated compounds.

Additional Analysis of Serum and Liver

Fluorimetric analyses of serum and liver vitamin A were performed on an Aminco-Bowman spectrophotofluorometer according to the method of Thompson et al. (10) with two modifications: 1) the liver was first macerated, than a 0.5 g aliquot was taken and homogenized with 0.5 ml 60% KOH in a Brinkman Polytron for 30 seconds before taking it through the rest of the procedure; and 2) the saponification was at 80° for 30 minutes.

Serum triglyceride analysis was performed using a colorometric procedure with Sigma triglyceride assay reagents (St. Louis, MO).

Analysis of the Fatty Acid Composition of Serum Lipids

Total lipid materials were extracted from the pooled rat serum according to the method of Folch et al. (11). Individual lipid classes were separated by the solvent system of petroleum ether:diethyl ether: acetic acid (85:15:1, v/v/v) on a silica gel thin-layer plate. Areas corresponding to cholesteryl ester, triglyceride, free fatty acid, and phospholipid were scraped off into ampules and fatty acid methyl esters were prepared by the method of Morrison and Smith (12). Analyses of fatty acid methyl esters by gas-liquid chromatography were carried out as previously outlined (13).

Experimental Design

Expt. 1: Thirty male Sprague-Dawley rats of average weight 143 g, were placed on the previously described diet (4) with retinyl acetate added at 1.2 R.E./g diet, a level suggested by the NRC to maintain adequate vitamin A nutriture. After 10 days, one group of six rats was randomly selected to continue on this diet. All other rats were fed a similar diet with supplemental retinoic acid incorporated at 100 µg/g diet in addition to the retinyl acetate. These diets were continued for 9 days until 0900 hour on the last day. All rats were then re-fed the diet containing only supplemental 1.2 R.E. retinyl acetate diet. At intervals during the next 54 hours, groups of rats were fasted for 6 hours and blood was sampled by heart puncture.

Expt. 2: Male Sprague-Dawley rats of average weight 146 g were maintained on a gel diet containing 1.2 R.E. retinyl acetate/g diet for six to seven days. Some rats were selected to continue on the retinyl acetate diet, while others were fed a diet containing 25 µg all-trans- retinoic acid/g of diet without supplemental retinyl acetate, for 12 to 13 days. At that time, rats were gastrically intubated under light ether anesthesia with 73 µg retinoic acid in 1.1 ml of corn oil at intervals which would allow for blood sampling at times up to 72 hours post-retinoic acid administration, but allowing the sampling to occur within the time period, 0930 to 1030 hours. Control rats were intubated with corn oil, 5 hours prior to sampling. After intubation, rats were fed the vitamin A-sufficient diet. All blood samples were taken in the nonfasted state.

Expt. 3: Male Sprague-Dawley rats of average weight 140 g were fed a gel diet containing 1.2 R.E. retinyl acetate/g of diet (RDA) for 8 to 9 days. A similar design to that employed in Expt. 2 was followed with the exception that all diets were supplemented with retinyl acetate at the basal level throughout the experiment, retinoic acid was fed for 8-9 days, control rats were intubated with corn oil 30 hours prior to sampling, and all rats were fasted 6 hours prior to blood sampling.

RESULTS

Expt. 1: Rats consumed an average of 16 g of control or retinoic acid-supplemented diets per day prior to the blood sampling period with an efficiency of 0.38 g weight gained per gram of food consumed. Serum was analyzed for total triglycerides and calculations were made to determine 1) if the reduction of blood triglyceride with the removal of retinoic acid from the diet followed a linear course; and 2) to determine how many hours were required for reduction to normal levels. A linear relationship between time after retinoic acid withdrawal and serum triglyceride levels, ($P \leq 0.05$) was not evident. It was apparent, however, that irrespective of the exact relationship between time and serum triglycerides, lipid levels were not different from controls at 30 hr post- retinoic acid feeding (Table 1).

Expt. 2: Rats consumed an average of 20 g of control or retinoic acid-supplemented diets per day prior to the blood sampling period with an efficiency of 0.33. Analysis similar to that in Expt. 1 revealed an inverse linear relationship between time after retinoic acid intubation versus serum triglycerides ($r=-0.73$, $P \leq 0.005$). Serum triglycerides were no longer elevated by 36 hours after the last administration of retinoic acid (Table 2). Liver vitamin A analysis revealed that the experimental groups had a lower concentration ($P \leq 0.05$) of vitamin A (52.93 ± 7.1 µg/g) than the control group (77.3 ± 11.7 µg/g). The lower concentration, however, is still an ample vitamin A liver store to insure adequate vitamin A status.

In addition to triglyceride analysis, fatty acid analysis of serum cholesteryl esters, triglyceride, phospholipid, and free fatty acid was performed on pooled rat sera from several groups (Table 3). As an overall

assessment, the percentage of palmitic and arachidonic acid was generally lower in most fractions while oleic and linoleic (18:1, 18:2) were higher in retinoic acid-fed rats. The fatty acid pattern returned to normal over time.

Expt. 3: Rats consumed an average of 21 g of control or retinoic acid-supplemented diets per day prior to the blood sampling period with an efficiency of 0.45. When serum triglyceride levels were analyzed statistically, it was found that by 24 hours after the last dose of retinoic acid, they were no longer elevated (Table 4). Also, there was an inverse linear relationship between serum triglycerides and time after dosing ($r=-0.683$, $P\leq0.05$). Serum retinoic acid reached a maximum at 12 hours after dosing and did not return to RDA-fed control levels until 72 hours ($P\leq0.05$). Serum retinoic acid levels were also time-related ($r=-0.519$, $P\leq0.005$). Serum retinol was depressed consistently during the interval studied until 72 hours, but did not exhibit a linear relationship over time, ($P\leq0.05$). When correlation coefficients were measured to determine the possible relationships between serum triglycerides, retinol and retinoic acid; retinoic acid levels were highly correlated with either triglycerides or retinol ($r=0.642$, -0.548; respectively, $P\leq0.05$). Retinol levels did not, however, correlate with serum triglycerides, ($P\leq0.05$). Fatty acid analysis indicated a shift to oleate and linoleate and a decrease in palmitate and arachidonate, similar to Expt. 2 (not shown).

DISCUSSION

The current series of experiments has attempted to investigate the relationship between serum triglycerides, retinol and retinoic acid following the cessation of feeding all-trans retinoic acid to rats. From the

TABLE 1
Effect of Retinoic Acid Withdrawal from the Diet Upon Serum Triglycerides in Rats (Experiment 1)

Hours Following Withdrawal of Retinoic Acid Supplemented Diets	Serum Triglycerides mg/100 ml[1]
6	359.0 ± 180.7[2,4]
18	343.7 ± 214.7[4]
24	335.9 ± 164.7[4]
30	219.4 ± 138.7
36	96.9 ± 9.8
54	72.6 ± 18.9
Control Rats[3]	98.2 ± 9.9

[1] Mean ± S.D.

[2] $N \geq 5$ for all groups.

[3] Average value for rats fed retinyl acetate diets and undergoing similar treatment to other rats.

[4] Elevated from control levels ($P\leq0.05$).

TABLE 2
Effect of Retinoic Acid Withdrawal from the Diet Upon Serum Triglycerides In Rats (Experiment 2)

Hour Following Intubation of a Single Dose of Retinoic Acid in Corn Oil	Serum Triglycerides mg/100 ml[1]
0	954.3 ± 243.9[2,4]
6	782.4 ± 224.7[4]
12	770.8 ± 330.0[4]
18	654.1 ± 424.4[4]
24	542.2 ± 219.1[4]
36	324.3 ± 71.4
54	278.9 ± 88.7
72	205.6 ± 49.3
Control Rats[3]	227.9 ± 101.6

[1] Mean ± S.D.

[2] $N \geq 5$ for all groups.

[3] Average value for rats fed retinyl acetate diets and undergoing similar treatment to other rats.

[4] Elevated from control levels ($P \leq 0.05$).

first experiment of the series, it was apparent that serum triglycerides did not return to normal until at least 30 hours after the retinoic acid was withdrawn from the diet of rats. The lack of significance to the relationship between serum triglyceride levels and the time after withdrawal suggested the need for a sampling procedure which would eliminate the effects of circadian rhythms. In the second experiment, intubation of a small dose of retinoic acid precisely marked the time the last retinoic acid was consumed, and the staggering of these intubations insured that all blood sampling would occur between 0930 and 1030. A significant linear relationship between serum triglycerides and time after withdrawal was observed with this design. A similar value of 36 hours for serum to return to normolipidemic levels after the last dose of retinoic acid was found for this experiment. An attempt was made in the last experiment to eliminate cyclicity, ensure precise knowledge of the time of the last dose, and to minimize serum triglycerides stemming directly from dietary sources. This was accomplished by following a similar regimen to the previous experiment, but also fasting the rats for 6 hours prior to sampling. When experiment 3 was analyzed, a similar degree of significance to Expt. 2 was associated with the relationship between triglyderide levels and time, with 24 hours required for resumption of normolipidemic levels.

TABLE 3
Fatty Acid Composition of Serum Lipid Fractions (Experiment 2)[1]

Lipid Fraction	Hours Following Retinoic Acid (RA) Withdrawal	Fatty Acid (%)[2] 14:0	16:0	16:1	18:0	18:1	18:2	20:4
Triglyceride	RDA control	1.0	20.7	6.0	0.6	33.3	36.7	1.3
	RA 0	1.3	19.4	7.3	0.6	34.1	37.3	trace
	" 6	1.2	21.3	7.2	trace	34.4	34.8	1.1
	36	0.9	21.8	7.6	trace	32.3	33.9	3.0
Cholesteryl Ester	RDA control	0.6	8.6	5.6	0.2	7.4	19.4	58.4
	RA 0	0.9	8.2	8.4	0.4	13.9	20.3	47.9
	" 6	0.5	9.1	7.2	trace	11.5	23.3	49.4
	" 36	0.3	7.6	6.8	trace	10.3	27.6	48.7
Free Fatty Acid	RDA control	2.8	25.4	6.5	8.0	23.5	25.4	8.3
	RA 0	2.4	24.8	7.6	5.1	28.0	27.6	4.5
	" 6	2.2	21.9	5.8	6.7	23.4	30.1	10.9
	" 36	2.7	25.2	6.2	8.8	22.3	26.3	9.6
Phospholipid	RDA control	0.4	27.4	0.2	18.7	12.0	20.5	20.9
	RA 0	0.3	21.4	2.0	17.1	14.3	26.4	18.4
	" 6	0.3	23.7	0.2	20.2	13.8	24.6	17.2
	" 36	0.3	23.9	0.4	21.0	11.9	23.2	19.3

[1]Rats fed RDA for vitamin A ± supplemental 25 µg retinoic acid/g diet for 8 days.

[2]All results expressed as an average area percent of triplicate analysis.

TABLE 4

Effect of Withdrawal of Retinoic Acid from the Diet Upon Serum Triglycerides, Retinoic Acid and Retinol in Rats (Experiment 3)

Hours Following Intubation of Retinoic Acid in Corn Oil	Serum Triglycerides[1] mg/100 ml	Serum Retinoic Acid µg/100 ml	Serum Retinol µg/100 ml
0	443.4 ± 199.1[2,4]	9.9 ± 3.2	67.3 ± 7.2[5]
3	622.2 ± 126.2[4]	37.2 ± 5.4[4]	53.3 ± 3.5[5]
6	843.8 ± 103.7[4]	42.2 ± 1.8[4]	65.0 ± 5.1[5]
12	557.5 ± 231.5[4]	45.8 ± 4.3[4]	67.9 ± 12.2[5]
18	429.9 ± 156.9[4]	20.0 ± 4.8[4]	62.4 ± 9.3[5]
24	254.6 ± 57.8	18.1 ± 4.2[4]	64.5 ± 5.7[5]
36	280.2 ± 184.7	9.3 ± 5.2	79.9 ± 9.8[5]
54	157.3 ± 12.5	15.5 ± 2.8[4]	72.6 ± 6.2[5]
72	163.6 ± 72.9	4.9 ± 2.6	83.0 ± 14.4
Control[3]	112.2 ± 69.5	not detectable[6]	93.6 ± 21.6

[1]Mean ± S.D.

[2]$N \geq 5$ for all groups.

[3]Average value for rats fed retinyl acetate diet and undergoing similar treatment to other rats.

[4]Elevated from control levels ($P<0.05$).

[5]Depressed from control level ($P\leq0.05$).

[6]Limit of detection is 1.0 µg/100 ml of serum.

One further observation concerns the alterations seen in the fatty acid composition of serum lipids. The increased percentage of 18:1 and 18:2 and decrease in 16:0 parallels the findings of Krause *et al.* (15) in dogs fed excess vitamin A. Although Krause fed an atherogenic diet to all dogs in contrast to the low-fat diets of our studies, they reported similar changes in 18:1 and 18:2 and 16:0 serum phospholipid and triglyceride fatty acids. On the other hand, Krause found increased 20:4 with vitamin A feeding while we have found a decrease in our studies. We can only speculate as to what the altered fatty acid profile indicates. An increase in 18:1 and 18:2 with a decrease in 20:4 and 16:0 could result from a decreased breakdown of triglycerides by LPL, with re-cycling of dietary 18:1 and 18:2 due to uptake by the liver from VLDL remnants. The lack of an increase in 16:0 would tend to contradict a hypothesis of excessive *de novo* fatty acid synthesis by liver. A decrease in 20:4 may result from a block in desaturation leading to this fatty acid.

Previous studies have suggested that both an increased rate of release of triglycerides from liver (5) and a decreased uptake by extra-hepatic sources (2, 5) resulted in the accumulation of triglycerides in the serum of

rats fed retinoic acid. Although the mechanism(s) have not been resolved, several hypotheses have been advanced. Erdman et al. monitoring lipid metabolism in post-mitochondrial liver homogenates observed an increased incorporation of cholesterol precursors into triglycerides in the presence of retinoic acid (16). Retinoic acid, although present in small amounts in some tissues (17) is not normally detectable in blood; but, if administered is bound to serum albumin (18). The depression in adipose and skeletal muscle lipoprotein lipase activity observed when retinoic acid is consumed (5) may also be a consequence of the unregulated delivery of retinoic acid to these tissues.

From the results of Experiment 3, it can also be observed that as the retinoic acid levels return to normal; so, too, does the serum triglyceride levels. Additionally, it is also apparent that as retinoic acid disappers from serum, serum retinol levels return to normal. The mechanism whereby retinoic acid administration depresses serum retinol is virtually unexplored. Serum retinol usually remains within a very narrow range in an animal with adequate zinc and protein nutriture and a moderate level of liver vitamin A stores. Recently, the central role of retinyl palmitate hydrolase in maintaining serum retinol levels has been suggested by Goodman et al. (19). Perhaps the hydrolysis of the retinyl ester to retinol may be altered by the presence of retinoic acid in the liver tissue. Alternatively, an effect may be occuring on either the synthesis or release of serum retinol-binding protein.

Only future studies can show whether these theories may prove valid. HPLC techniques for measuring vitamin A and retinoid tissue levels have only recently allowed for establishing their presence or absence in various tissues and the changes which occur with retinoid feeding.

REFERENCES

1. MISRA, U. K. Effect of intramuscular administration of retinol on plasma lipids of rats. Agr. Biol. Chem. 32, 707-710, 1968.

2. SINGH, M. and SINGH, V. N. Fatty liver in hypervitaminosis A: synthesis and release of hepatic triglycerides. Am. J. Physiol. 234, E511-E514, 1978.

3. GERBER, L. E. and ERDMAN, J. W., JR. Effect of retinoic acid and retinyl acetate feeding upon lipid metabolism in adrenalectomized rats. J. Nutr. 109, 580-589, 1979.

4. GERBER, L. E. and ERDMAN, J. W., JR. Comparative effects of all-trans and 13-cis retinoic acid administration on serum and liver lipids in rats. J. Nutr. 110, 343-351, 1980.

5. GERBER, L. E. and ERDMAN, J. W., JR. Hyperlipidemia in rats fed retinoic acid. Lipids, 1981 (in press).

6. KATZ, R. A., JORGENSEN, H., and NIGRA, T. P. Elevation of serum triglyceride levels from oral isotretinoin in disorders of keratinization. Arch. Dermatol. 116, 1369-1372, 1980.

7. KEILSON, B., UNDERWOOD, B. A. and LOERCH, J. D. Effects of retinoic acid on the mobilization of vitamin A from the liver in rats. J. Nutr. 109, 787-795, 1979.

8. BESNER, J.-G. and LECLAIRE, R. High-performance liquid chromatography of 13-cis-retinoic acid and of endogenous retinol in human plasma. J. Chrom., Biomed. Appl. 183, 346-351, 1980.

9. ITO, Y. L., ZILE, M., AHRENS, H. and DELUCA, H. F. Liquid-gel partition chromatography of vitamin A compounds; formation of retinoic acid from retinyl acetate in vivo. J. Lipid Res. 15, 517-524, 1974.

10. THOMPSON, J. N., ERDODY, P., BRIEN, R. and MURRAY, T. K. Fluorometric determination of vitamin A in human blood and liver. Biochem. Med. 5, 67-89, 1971.

11. FOLCH, J., LEES, M. and SLOANE STANLEY, G. H. A simple method for the isolation and purification of total lipides from animal tissues. J. Biol. Chem. 226, 497-509, 1957.

12. MORRISON, W. R. and SMITH, L. M. Preparation of fatty acid methyl esters and dimethylacetate from lipids with boron fluoride-methanol. J. Lipid Res. 5, 600-608, 1964.

13. CHO, B.H.S. and KUMMEROW, F. A. Lipid composition and metabolic activity of the microsomal fractions from the arterial and liver tissues of swine. Biochem. Med. 20, 267-278, 1978.

14. NATIONAL RESEARCH COUNCIL. Nutrient requirements of domestic animals. Nutrient requirements of laboratory animals. Third revised edition. National Academy of Sciences, Washington, D.C., 1978, pp. 20-21.

15. KRAUSE, R. F., PALLOTTA, M. A. and YODER, I. I. Lipid alterations in beagles produced by atherogenic stress and vitamin A. J. Atheroscler. Res. 8, 277-289, 1968.

16. ERDMAN, J. W., JR., ELLIOT, J. G. and LACHANCE, P. A. The effect of three forms of vitamin A upon in vitro lipogenesis from three cholesterol precursors. Nutr. Rep. Int. 16, 47-57, 1977.

17. KLEINER-BOESSALER, A. and DELUCA, H. F. Formation of retinoic acid from retinol in the kidney. Arch. Biochem. Biophys. 142, 371-377, 1971.

18. SMITH, J. E., MILCH, P. O., MUTO, Y. and GOODMAN, D. S. The plasma transport and metabolism of retinoic acid in the rat. Biochem. J. 132, 821-827, 1973.

19. HARRISON, E. H., SMITH, J. E. and GOODMAN, D. S. Unusual properties of retinyl palmitate hydrolase activity in rat liver. J. Lipid Res. 20, 760-771, 1979.

NUTRITION RESEARCH, Vol. 1. pp. 519-524, 1981
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EFFECT OF SPINAL CORD TRANSECTION ON PLASMA AND HEPATIC LEVELS OF CERTAIN ESSENTIAL ELEMENTS AND VITAMINS IN RATS*

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ABSTRACT

This study was conducted to determine whether spinal cord transection produces nutritional deficiency states in certain essential elements and water soluble vitamins in the rat. Spinal cords of female rats were severed at the T-9 level and these animals were matched and pair-fed to control animals that had undergone sham surgery. Following periods of 60, 90 and 180 days, animals were sacrificed and their plasma and livers assayed for selenium, manganese, zinc and magnesium and eight water soluble vitamins. The results indicate that spinal cord injury did not cause deficiencies in those nutrients studied. Instead the cord transection produced accumulations in the liver of all the elements and six of the vitamins studied. No explanation for these accumulations is known at this time.

Key Words: spinal cord injury, rats, elements, vitamins

INTRODUCTION

Through clinical experience with patients with spinal cord injury, it is well known that these handicapped persons suffer from several maladies which are secondary to the basic lesion. It is established that paraplegics and quadriplegics endure problems involving kidney, skin, intestines, bladder and bones. To date the basic cause of these problems is not known and no working hypothesis has been advanced to arrive at the etiology of these conditions.

A practical hypothesis has been formulated in this laboratory which proposes that some of the secondary problems suffered by spinal cord patients may be due to poor nutrition. It is entirely feasible that the spinal cord lesion may lead to inadequate absorption of nutrients essential to the organism.

*This research was partially supported by a grant from the Technology and Research Foundation of the Paralyzed Veterans of America.

To test this hypothesis, this study was designed to determine whether deficiency states of certain essential nutrients were produced with time following spinal cord injury in animal models. Specifically, plasma and liver levels of zinc, manganese, selenium and magnesium and some water soluble vitamins from whole blood were monitored in a time course study following spinal cord transection in rats and the values compared to those in pair fed, control animals monitored over the same time period.

MATERIALS AND METHODS

Female, Sprague-Dawley rats approximately 250 g were chordotomized according to the method of Feringa, et al. (1). This involved anesthetizing the animal with pentabarbital and exposing the spinal cord beneath the dura in the mid-thoracic area. The spinal cord was then transected by a small scissors at the T-9 level and the operative site closed. Another group of female rats of the same age and weight were sham operated. Surgery was carried out through the point of cutting through the dura but the cords were not transected. Postoperatively the bladders of the transected animals were emptied every 8 hours by the Credé method until reflex control of the bladder returned in approximately 2 weeks. Two days following the operative procedures, the animals were divided into pairs and the control animals were pair-fed to the dietary intake of the transected animals using Purina rat diet. All animals were given water ad libitum. Six pairs of animals were killed by exsanguination at each of 60 day, 90 day and 180 day time periods. Plasma was prepared from an aliquot of the blood samples and stored at -70° and the livers of the animals were freeze-clamped and stored at -70°. The remaining aliquot of whole blood was stored at -70° and later used for the vitamin analyses.

Zinc and magnesium levels in both plasma and liver were measured by digesting these samples in concentrated nitric acid, diluting to a known volume and estimating the amounts by atomic absorption spectrophotometry (2). Selenium and manganese levels in plasma and liver were measured by the neutron activation methods of Hahn, et al. (3,4).

Whole blood and hepatic levels of the water soluble vitamins, thiamine, riboflavin, vitamin B_6, nicotinic acid, pantothenic acid, biotin, vitamin B_{12} and folic acid were assayed by the methods described by Baker and Frank (5).

Since spinal cord transection produced some differences in body weights between the control and experimental animals, levels of trace elements and vitamins were expressed on the basis of body weights (6).

RESULTS

The plasma values of the elements listed in Table 1 for the time periods following spinal cord transection suggests that the severing of the spinal cord does not impede the absorption of elements in the intestine and allows the same availability to these elements as seen in control animals.

TABLE 1

Plasma and Hepatic Levels of Trace Elements[1] Following Spinal Cord Injury (SCI) in Rats

ELEMENT	LIVER (µg/total liver per 100g body weight)		
	60 days	90 days	180 days
SELENIUM			
Controls	3.7 ± 0.45	3.5 ± 0.14	4.0 ± 0.15
SCI	4.8 ± 0.19[2]	4.8 ± 0.45[3]	4.6 ± 0.20[2]
MAGNESIUM			
Controls	711 ± 60	702 ± 25	786 ± 43
SCI	951 ± 40[3]	848 ± 25[4]	894 ± 43[5]
ZINC			
Controls	82 ± 6.8	81 ± 3.2	96 ± 6.4
SCI	115 ± 13.4[2]	99 ± 2.8[3]	105 ± 11.0[5]
MANGANESE			
Controls	5.0 ± 0.56	5.1 ± 0.31	6.3 ± 0.61
SCI	7.3 ± 0.41[2]	6.3 ± 0.26[4]	9.2 ± 0.58[3]

ELEMENT	PLASMA		
	60 days	90 days	180 days
SELENIUM		µg/ml	
Controls	0.27 ± 0.02		0.37 ± 0.02
SCI	0.30 ± 0.03[5]		0.31 ± 0.02[5]
MAGNESIUM		mg/dl	
Controls	2.13 ± 0.11	2.26 ± 0.18	2.88 ± 0.30
SCI	2.06 ± 0.12[5]	2.25 ± 0.16[5]	2.68 ± 0.34[5]
ZINC		mg/dl	
Controls	0.14 ± 0.01	0.17 ± 0.02	0.17 ± 0.01
SCI	0.14 ± 0.01[5]	0.18 ± 0.02[5]	0.18 ± 0.01[5]

[1]Values are means from 6 animals ± SEM

[2]When compared to comtrols by paired student t test: $p < 0.05$

[3]$p < 0.01$

[4]$p < 0.001$

[5]Not significant

The liver levels of the four elements studied (selenium, magnesium, zinc and manganese) show that spinal cord injury does not produce a deficiency of essential elements in the liver and paradoxically, appears to produce an accumulation of these elements in hepatic tissue.

TABLE 2

Whole Blood and Hepatic Levels[1] of Vitamins Following Spinal Cord Injury (SCI) in Rats

VITAMIN	LIVER		
	60 days	90 days	180 days
PANTOTHENIC ACID		(µg/total liver per 100g body weight)	
Controls	154 ± 12	159 ± 16	45 ± 3
SCI	208 ± 18[2]	191 ± 24[6]	61 ± 4[4]
BIOTIN		(ng/total liver per 100g body weight)	
Control	469 ± 84	506 ± 48	177 ± 15
SCI	673 ± 126[2]	674 ± 71[4]	234 ± 33[5]
VITAMIN B12			
Control	267 ± 15	283 ± 48	231 ± 41
SCI	423 ± 52[4]	329 ± 55[6]	406 ± 73[6]
FOLIC ACID		(µg/total liver per 100g body weight)	
Control	47 ± 5	38 ± 3	39 ± 4
SCI	65 ± 4.5[3]	51 ± 3[4]	44 ± 5[6]
THIAMINE			
Control	35 ± 3.9	33 ± 3.2	34 ± 3.1
SCI	44 ± 2.1[2]	36 ± 3.8[6]	35 ± 3.4[6]
RIBOFLAVIN			
Control	97 ± 6	92 ± 5	102 ± 7
SCI	121 ± 9[6]	117 ± 12[3]	125 ± 8[2]
VITAMIN B6			
Control	21.9 ± 2	22.1 ± 2	13.5 ± 1
SCI	25.4 ± 2[6]	24.6 ± 2[6]	14.9 ± 1[6]
NICOTINIC ACID			
Control	292 ± 26	251 ± 26	253 ± 30
SCI	401 ± 46[6]	329 ± 46[6]	307 ± 21[6]

(continued)

TABLE 2 (continued)

Whole Blood and Hepatic Levels[1] of Vitamins Following Spinal Cord Injury (SCI) in Rats

VITAMIN	WHOLE BLOOD		
	60 days	90 days	180 days
PANTOTHENIC ACID		ng/ml	
Controls	532 ± 43	461 ± 32	374 ± 8
SCI	533 ± 54[6]	376 ± 55[6]	374 ± 9[6]
BIOTIN			
Control	9.9 ± 0.8	9.5 ± 0.9	1.2 ± 0.2
SCI	10.0 ± 1.4[6]	13.4 ± 2.2[6]	1.6 ± 0.2[6]
FOLIC ACID			
Control	834 ± 56	678 ± 52	490 ± 25
SCI	645 ± 94[6]	650 ± 49[6]	531 ± 34[6]
THIAMINE			
Control	266 ± 12	300 ± 20	507 ± 38
SCI	299 ± 15[6]	315 ± 22[6]	479 ± 43[6]
RIBOFLAVIN			
Control	128 ± 10		143 ± 29
SCI	120 ± 2[6]		157 ± 7[6]
VITAMIN B6			
Control	99.5 ± 9	94 ± 7	43 ± 3
SCI	90.8 ± 4[6]	126 ± 13[3]	44 ± 4[6]
NICOTINIC ACID		µg/ml	
Control	9.4 ± 0.27	9.5 ± 0.20	11.6 ± 1.5
SCI	9.5 ± 0.50[6]	9.8 ± 0.44[6]	10.9 ± 0.6[6]

[1]Values are means from 6 animals ± SEM

[2]When compared to the controls by paired student t test: $p < 0.05$

[3]$p < 0.02$

[4]$p < 0.01$

[5]$p < 0.001$

[6]Not significant

The changes produced by spinal cord transection in the plasma and hepatic levels of essential elements also appear to hold true for most of the water soluble vitamins studied. In these cases, the cordotomy did not affect absorption of the vitamins with the levels remaining the same in control and experimental animals throughout the 6 month study. But, for the most part, spinal cord injury appears to produce an accumulation of most of the water soluble vitamins in liver. Pantothenic acid and biotin show a hepatic accumulation throughout the total 180 day study. Vitamin B_{12}, folic acid and thiamine show early accumulations with return to control levels with time. Riboflavin shows a latent accumulation but vitamin B_6 and nicotinic acid levels in liver were not affected by the spinal cord lesion.

DISCUSSION

The most interesting observation found in this study is that total spinal cord severance at the T-9 level, which produced total and permanent loss of use of the hind limbs in the rat, did not produce deficiencies within a wide range of essential nutrients. The elevated hepatic levels in the experimental animals of the elements and vitamins studied suggests that changes may occur in certain mechanisms to bring about these results. A few of the possible changes are: (a) Increased absorption of the nutrients in the intestines; (b) Lack of hepatic transport systems for nutrients from the liver, and (c) Decreased need of hepatic components in the experimental animals due to lesser physical activity in these animals. The normal levels of elements and vitamins in the blood of the experimental animals at the various periods throughout the course of the study, however, does not support any one of these possibilities as a mechanism. Therefore, no clear explanation for the accumulation of elements and vitamins in the livers of experimental animals is evident at this time.

Aside from complete loss of use of the hind limbs in the experimental rats in this study, these animals were all in the state of well-being throughout the 180 day period and did not appear to be in any nutritional deficiency. These animals did not develop any problems related to skin, bone, kidney or intestine and hence it would seem that the effects of spinal cord transection in the rat are quite different from those observed in humans.

ACKNOWLEDGEMENT

The authors wish to thank Mr. Roger Lainson and Mr. William Baier of the Great Plains Chapter of the Paralyzed Veterans of America for their assistance and encouragement during the course of this study.

REFERENCES

1. FERINGA, E.R., GURDEN, G.G., STRODEL, W., CHANDLER, W. and KNAKE, J. Descending spinal cord motor tract regeneration after spinal cord transection. Neurology, 23: 599-608, 1973.

2. Perkin-Elmer Technical Bulletin, Analytical Methods for Atomic Absorption Spectrophotometry. Perkin-Elmer Corp. Norwalk, Conn., September, 1968.

3. HAHN, K.J., WILLIAMS, R.V., BURCH, R.E., SULLIVAN, J.F. and NOVAK, E.A. Determination of serum selenium by means of solvent extraction combined with activation analysis. J Lab Clin Med 80: 718-722, 1972.

4. HAHN, K.J., TUMA, D.J. and SULLIVAN, J.L. Rapid and simple continuous radiochemical separation of copper, magnesium, zinc and manganese in biological materials. Anal Chem 40: 974-976, 1968.

5. BAKER, H. and FRANK, O. Clinical Vitaminology, Methods and Interpretation, Interscience Publishers, New York, London, Sidney, Toronto, 1968.

6. BARAK, A.J., TUMA, D.J. and SORRELL, M.F. Current considerations regarding reference units in the expression of biomedical data. Life Sci 17: 253-256, 1975.

NUTRITION RESEARCH, Vol. 1, pp. 525-550, 1981
0271-5317/81/050525-26$02.00/0 Printed in the USA.
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PERIODICITY OF EATING: IMPLICATIONS FOR HUMAN FOOD CONSUMPTION

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ABSTRACT

The literature review summarizes pertinent research on the effects of meal frequency. Metabolic adaptation including morphological and physiological changes in laboratory animals is reported. Earliest research supported the hypothesis that meal frequency did influence metabolic functions and thereby affected weight gain or loss. More recent research in laboratory animals indicates that erroneous conclusions may have been drawn by misinterpretation of research results, and that meal frequency does not influence changes in body fat nor weight. Implications of animal research to man is reported and the contemporary meal habit that includes snacking is discussed.

KEY WORDS: Meal frequency, metabolic adaptation, snacking.

INTRODUCTION

Was man originally a "nibbler" as were the slender beauties of Bali, mentioned by Harvard scientist, Dr. Frederick Stare? This reknowned former chairperson of the Department of Nutrition at the Harvard School of Public Health once stated, "I never saw a fat woman in Bali. These people appeared to be eating all the time, but only a handful at a time (1)." Or is meal-eating with its presumed unwelcome sequelae the product of the process of civilization leading to the American custom of the evening meal wherein the family consumes 50 to 75 percent of the daily calories? (2)

Anthropologists tell us that for ancient man a pattern with occasional food intake in larger portions predominated - dictated by accessibility of food made available following a successful hunt or food collecting expedition. With the advent of agriculture and technology came the knowledge of methods for food storage and preservation, thus giving rise to more continuous eating habits. The pattern whereby the contemporary American or western European individual generally consumes two to three meals per day may be compared to ancient man who appeared "naturally adapted" to intermittent food intake. Animals living in the wild must consume food when it is made available and maximize the efficient utilization of the food they get. Holecková compared the domesticated laboratory rat with its wild counterpart and demonstrated that the laboratory rat, once adapted to intermittent fasting, resembled the typical wild rat particularly in its capacity for hyperphagia (3). The temptation is great for harmonizing this

assumption with epidemiological data of pathogenicity in cultures that adhere to a single meal-eating dietary pattern. Statistics may be interpreted so that it appears many of "societal ills," for example diabetes, cardiovascular disease and obesity, proceed parallel with the degree of civilization and standard of living.

Evidence exists that meal-eating does affect metabolism. Work with animals has shown that consuming total food intake in fewer feedings induces adaptive changes that include: alterations in overall energy metabolism, more rapid intestinal absorption of glucose and fat, increased glucose synthesis, enhanced lipogenesis, and elevated blood serum cholesterol (4). These changes correspond in man with tendensies for greater body weight, increased skinfold thickness, diminished glucose tolerance and hypercholesterolaemia (5).

In light of experimental evidence, it seems paradoxical that we hear fervent excoriation among some nutritionists and "health enthusiasts" for the increasing trend of consuming fewer large meals and including several small snacks throughout the day. Instead we see the habit of eating between meals referenced as being undesirable (6). While some snacks of low nutrient quality may have limited role in diets of individuals with low caloric requirement (7), the principle behind snacking as a mode of nutrient consumption should be evaluated in view of subsequent physiological adaptation and psychological "benefits and risks".

In studying the metabolic and physiologic makeup of man, the scientist is encumbered in the realm of ethics that restrict the use of human subjects in classical scientific research. Thus, theoretical conclusions regarding the metabolic effect of feeding frequency in laboratory rats must be extrapolated to man with his distinctive physiologic characteristics. One further handicap is that in a rat model, a researcher must ignore the psychologic aspect of food and the diet that exists for man. This omission enhances the prospect for misinterpretation when results from animal studies are extrapolated to man. Disadvantage with lack of human research data is clear and reliance on animal data to make inference for human consumption patterns is undesirable. Numerous inconsistencies exist among animal data which will be illustrated in this report. Effort is made to allude to the patent need for emphasis on human research in the area of meal-feeding and its metabolic implication.

Laboratory models.

Animals historically used by researchers exploring the effects of meal frequency includes a wide gamet of species--from rats to ruminants and chickens to cats. Majority of experimental research has focused on various strains of the laboratory rat as the animal model. Domestic rats are by nature "nibblers" eating small amounts of food at frequent intervals and reaching maximal intake during the night. For experimental purposes, access to food is restricted to 1-2 hr/day (7), or rats are fed intermittently only three days per week alternating with one - to two day periods of fasting (8). The animal soon learns to ingest large amounts of food when it is made available and thus becomes a "gorger." The total food intake may be the same or is even reduced as the rat usually does not fully compensate for the periods of food deprivation (9). Rats trained to consume their food in a short period of time each day usually ingest only 60 to 80 percent of the nutrients consumed by control rats given free access to food (10). Since animals may refuse to consume their normal food intake at a single sitting, researchers often resort to another experimental approach, e.g., force feeding by means of a gavage (11, 12), in order to study the effects of ingesting large amounts of food.

Morphological changes in meal-fed animals.

Studies involving the effect of intermittant fasting over extended periods in rats have shown a variety of morpholgical adaptations (8). Changes include enlargement of both the stomach and the glandular portion of the stomach with meal-feeding of 17 weeks or longer (13). Total body weight for the rat was reduced, yet absolute weight of the stomach and its portions was increased with a marked hypertrophy of the mucosa and musculature--and particularly striking was the increased size of the prepyloric region. The capacity of this organ for food storage is remarkable, and adapted animals have been observed to have considerable amount of food still remaining in this region even after a subsequent 24 hour fast. Holecková and Fábry (8) observed that another portion of the digestive tract, the small intestine, responded in a manner analogous to the stomach. The small intestine did not lose weight during the first six weeks of experimental feedings, although body weight during this period declined 30 percent compared with ad libitum-fed controls. In another study, Fábry (14) observed a 16 percent increase in length of the small intestine with meal feeding and similarly, organ weight of meal-fed, intermittently fasted animals was 68 to 89 percent greater than for ad libitum-fed controls.

Concurrent with morphologic changes reported in meal-fed rats, histologic examinations indicate that intestinal tissue adapts to meal-eating. Specimens from the oral intestinal wall, and distal portions of the jejumun and the distal part of the ileum from rats subjected for 16 weeks to intermittant feeding revealed that area calculated as the annular space was 18 percent greater for adapted versus control animals (15). Pathologic changes, including possible differences in size of mitochondria, Golgi bodies or enterocytes were not observed. Therefore, it appears that enlargement of the intestine with adapted animals corresponds to a greater increment of functional tissue within the organ. Consistant with enhanced functionality of the tissue, Pocknee and Heaton (16) observed that weight of the liver, kidneys and femur were increased in young meal-eating rats compared to continuously-fed control animals. These differences persisted even into the adult life of the animals.

Physical adaptations lead to enhanced nutrient absorption.

It appears that nature has provided animals with the extraordinary ability to adapt to diverse conditions imposed on a species, and numerous physiologic changes make it possible for an animal to survive on a restricted feeding schedule. The increased surface area of the enlarged gastrointestinal tract of meal-fed animals allows for enhanced absorption of nutrients. Increased absorption may be an intermediate link in the mechanism that permits the adapted animal to form metabolic reserves from ingested food.

Researchers have attempted to explain the effect of meal-feeding on physiological systems by investigating possible primary factors. Enzymes act as critical components in determination of reaction rates within biological systems including nutrient digestion and absorption. Increased activity of the enzyme sucrase, responsible for the breakdown of sucrose to its simple constituents of glucose and fructose, makes available a greater amount of glucose for absorption, given that a dietary source of the complex carbohydrate is provided. Glucose absorption has been the subject of considerable investigation by numerous researchers in relation to meal frequency. Stevenson and Fierstein (17) investigated feeding frequency and nutrient absorption rate in relation to circadian rhythms in synthesizing enzymes required for the active transport of carbohydrate. Researchers found that sucrase activity was greatest just prior to feeding and transport activity peaked near the end of the feeding period. Times with peak absorption corresponded with the time that food consumed was presented in the small intestine. It thus appears that rhythm and physiological function are

dictated by time of eating.

Furthermore, the research team of Kujalová and Fábry (18) showed that after 6 weeks on a meal-feeding regimen there was a marked increase in glucose absorption rate compared with controls. Chakrabarty and Leveille (19) confirmed the previous report when they observed an enhanced glucose absorption rate with meal-feeding. Researchers attributed the increase directly to the increase in intestinal weight of the meal-fed rats. Analyses focused on the problem whether chronic reduction of food supply alone, which reflected a probable decreased food intake for meal-fed animals, could serve as stimulus for enhanced absorption (18). Research reports from the 1940's tend to suggest the alternate, that caloric deficiency leads to reduced nutrient absorption (20, 21). Therefore, in 1960, Kujalová and Fábry (18) studied rats that had been first adapted to intermittent starvation and then realimented to one of two regimes. One group was offered food ad libitum while the other was fed iso-calorically with a set of control rats. If diets that included intermittent starvation were replaced by simple caloric reduction to 60 percent of the intake of controls then a rapid decrease in glucose absorption occurred. Glucose absorption rate was significantly lower than for ad libitum-fed controls after 4 weeks of experimental feeding (Figure 1). This confirmed the notion that general caloric undernutrition leads to a reduced glucose absorption. Later research by Fábry (22) supported earlier results and animals fed for a period of 6 weeks with a daily ration lower in total calories than ad libitum-fed controls experienced a 25 percent decline in glucose absorption. It is apparent that stimulus leading to the enhanced intestinal absorption of glucose in intermittently fasting rats is not the reduction of calorie intake per se, but is rather the effect of periodic hyperphagia whereby a large amount of food is consumed at one time.

The enhanced nutrient absorption associated with meal-eating observed with glucose should be indicated for amino acid absorption also. However, Kojalová and Fábry (18) investigated the effect of feeding frequency on amino acid absorption and showed no significant differences when intermittently starved animals were compared with controls after 6 weeks of experimental treatment. The author speculated that differences might be observed after a prolonged period of adaptation on basis of a subsequently increased intestinal surface area with meal-feeding.

That enhanced glucose utilization may not necessarily follow enhanced absorption has been investigated. Leveille (23) showed that blood glucose levels differed for fasted meal-fed and nibbling rats after force feeding 2 gm of glucose. Blood glucose increased to a lesser extent in meal-fed animals compared to nibblers in spite of the fact that glucose was being absorbed more rapidly. Fábry (24) and Tepperman (25) found similar results to support the theory that tissues of meal-fed animals utilize glucose more rapidly than do those of the nibbling rat. Fábry (9) and others (5, 26, 27) have since reported an impaired glucose tolerance for humans with a decreased meal frequency, but other researchers have failed to substantiate these results in animal models (28, 29, 30).

Increase in glucose absorption and utilization cues lipogenesis activity.

Leveille (23) suggested that the reason for enhanced ability for glucose utilization with meal-feeding was to allow for considerable portions of glucose to be converted to storage forms. Storage forms were primarily glycogen and lipid. Evidence of augmented biochemical activity following meal-eating was demonstrated by Leveille (31) with the existence of a respiration quotient (RQ) in excess of unity persisting for up to 8 hours after initiation of the daily meal in meal-fed animals. Elevated RQ indicates that glucose is the major oxidative fuel and that lipogenesis is proceeding at

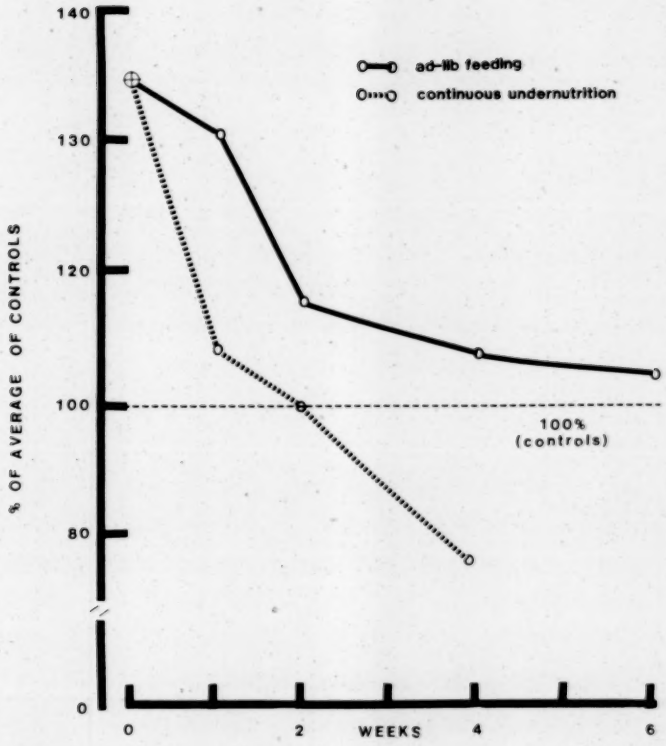

FIG. 1

Regression of adaptive increase of intestinal absorption in intermittant fasting (0) in the course of subsequent ad libitum feeding or continuous undernutrition. Values are averages of groups compromising 5-8 animals, expressed as percentage of the average. Reproduced from Fabry (18).

a rapid rate. Leveille's reports corroborated those of Tepperman et al. (7) who showed that rapid lipogenesis is limited to an 8 hour period following meal initiation. The rapid influx of nutrients incurred as a result of enhanced intestinal absorption stimulates biochemical changes of the intestinal mucosa and affects utilization of nutrients and their metabolites. Thus, the meal-fed animal becomes more efficient in converting carbohydrate to storage materials (19). Most marked changes in the rat occurred in adipose tissue since adipose is the primary site of lipogenesis in the rat (32). Adipose lipogenesis accounted for 95 percent of the total fatty acids synthesized in the meal-fed rat (33).

The above set of changes identified in both liver and adipose tissue have been collectively designated by Tepperman and Tepperman as "adaptive lipogenesis" (34). Metabolic changes in meal-fed animals leading to this phenomonon are primarily those involving carbohydrate metabolism. That activity of the pentose pathway enzymes is enhanced by meal-feeding is well substantiated (12, 35, 36). Leveille (23) has studied activity of a number of

enzymes in adipose tissue of meal-fed and nibbling animals. These enzymes have been considered in three broad groups. Categorically, these may be classified as (a) those involved in phosphorylation of glucose and its conversion to alpha-glycerophosphate and pyruvate, (b) enzymes involved in fatty acid synthesis, and (c) enzymes involved in the generation of NADPH for the support of fatty acid biosynthesis. Activities of hexokinase, pyruvate kinase, alpha-glycerophosphate, acetyl-CoA carboxylase and carboxylase were significantly elevated in adipose tissue of meal-fed as compared with nibbling rats. It is recognized that glucose metabolism is initiated by phosphorylation of the substrate. Enhanced activity of key phosphorylating enzymes, glucokinase and hexokinase, are therefore of interest in relation to the initiation of a more rapid utilization of glucose in meal-fed animals.

Pyruvate kinase is an enzyme of interest since it is key in the glycolytic pathway. The enzyme alpha-glycerophosphate dehydrogenase is responsible for supplying glycerophosphate for the synthesis of triglyceride. Chakrabarty and Leveille (19) assayed activity of these two enzymes in liver, adipose and muscle of meal-eating and nibbling animals. Both of these enzymes registered activity that was significantly higher in adipose tissue of meal-fed rats compared with nibbling rats. Activity of these enzymes in either liver or muscle, however, was not influenced by method of feeding. In a more recent study, Romsos and Leveille (36) identified intestinal pyruvate kinase existing at similar levels of activity in both meal-fed and nibbling rats. Hahn and Skala (37) have previously shown that ingestion of a high fat diet depressed pyruvate kinase activity in the intestine, but that meal-eating had only a slight influence on intestinal pyruvate kinase activity.

Pyruvate carboxylase activity was also elevated in meal-fed animals. The team of Chakrabarty and Leveille (19) showed that activity for this enzyme was three to four times greater in liver tissue compared to that in nibbling rats and was four to five times higher for adipose. Phosphoenolpyruvate (PEP) carboxylase activity was accelerated only slightly and only in liver of meal-fed animals -- not in adipose.

What is the significance of enhanced enzyme action within an organ? Research substantiating the selectively enhanced activity of some enzymes in adipose but not in other tissue indicates that the effect of meal-eating may depend on the biochemical function of the particular tissue in question. Ratios for activity of various enzymes in liver to that in adipose tissue are significant in establishing their relative roles in lipogenesis and glucogenesis. It is clearly apparent from experimental data that the biological system of the meal-fed individual has been "geared" for lipogenesis to occur at a rapid rate. Adipose tissue is the primary site of lipogenesis in the rat and it has shown the greatest ability to adapt to imposed meal-feeding conditions (33, 38-41). Liver tissue has only a minor role in total fatty acid synthesis in the rat. Enzymes involved in gluconeogenesis show an accelerated activity in hepatic tissue for meal-eaters relative to nibbling animals. Meal-eating animals have need for a supply of glucose between meal periods with long periods of deprivation from dietary glucose. Enhanced activity of PEP-carboxylase, for example, indicates that gluconeogenesis is proceding at a rapid rate in meal-fed individuals. The enzyme shows no increase in adipose since adipose is not involved in gluconeogenesis.

Another catagory of enzymes to show enhanced activity with meal-eating are those involved with supplying reduced NADPH necessary for fatty acid synthesis. Pyruvate carboxylase and malic enzyme are two such enzymes and both have been shown to markedly increase with meal feeding (23, 24).

The pentose pathway, or hexose monophosphate shunt, is the other potential source for reduced NADPH essential for fatty acid synthesis. Enhanced

activity of glucose-6-phosphate dehydrogenase in the pathway has been indicated by Leveille (33). G-6-P signifies a crossroads in glucose metabolism and is involved in glycogen formation, gluconeogenesis, glycolysis, or in the pentose pathway. Accelerated activity of G-6-P dehydrogenase would influence the preference that the compound be rendered available for production of NADPH (42) and could subsequently go to support fatty acid synthesis.

The sequence involved? Which comes first: The "chicken or the egg"?

The positive correlation between lipogenic capacity and pentose pathway dehydrogenase activity has raised questions regarding their relative time sequence for initiation with meal-feeding. It has been suggested that an enhanced rate of fatty acid synthesis stimulates an increase in pentose pathway activity. Results of Tepperman and Tepperman (43) confirm this hypothesis. They observed increased lipogenesis preceding accelerated dehydrogenase activity of the pentose pathway in livers of fasted-refed rats. Leveille (23) showed that after 5 days of meal-feeding rats, lipogenesis was increased significantly whereas activity of the pentose pathway dehydrogenase and malic enzyme was actually below that in nibbling rats. Enzyme activity did not attain a level exceeding that of controls until day nine of the meal-feeding regimen. Consequently, it was concluded that the increase in lipogenesis was clearly not a response to the enhanced activity of related enzymes. Foster and Srere (44) have substantiated this conclusion with evidence regarding hepatic citrate-cleavage enzyme activity. They found that an enhanced activity induced by refeeding following a fast was separated from the lipogenic response by a significant time lag. It is conceivable that an accelerated rate of lipogenesis would oxidize a considerable amount of NADPH, thereby increasing the concentration of $NADP^+$. This, in conjuction with the presence of additional required components, would increase the flux of substrate through the enzymatic processes and thereby trigger enhanced enzyme activities (23, 45, 46).

Researchers continue to question.

That lipogenesis is more rapid in gorging rats than in nibbling rats has been well substantiated (33, 35, 47, 48). Results of numerous studies suggest that there is a more rapid induction of lipogenic mechanisms in trained meal-fed animals relative to their nibbling counterparts and that meal-fed animals show enzymatic adaptation during the training period. Recently, Baker et al. (49, 50) suggested that changes occurring in normal nibbling animals had not received adequate attention and attempted to substantiate his theory doubting that observed differences did in fact exist. Baker (49) compared rates of fatty acid synthesis from glucose-C in untrained mice fed ad libitum with those that had undergone various periods of fasting with refeeding. Baker and co-workers found that meal-fed mice had rates of fatty acid synthesis that exceeded those for nibbling animals. Yet he furthermore inferred from results that the rate of lipogenesis may be accelerated or decreased both during and after ingestion of food much more rapidly than had been surmised from earlier reports. In a later study done in 1976, Baker (50) compared average rates of exogenous glucose-C conversion to whole body and total lipid fatty acids and failed to show a difference in rate of glucose-C incorporation.

In efforts to explain Baker's reports that clearly contradict those of other investigators, it seems practical to suggest that the composition of the experimental diet has significance on observed results. The ratio of major nutrients in the diet exerts a marked influence on metabolic processes associated with their utilization -- beginning with digestion and absorption from the intestinal tract and ending with dissimulation, interconversion, deposition of reserves, with final oxidation to yield energy (22). It has

been shown that a high carbohydrate intake increases lipogenesis from carbohydrate precursors, glycogen synthesis and glucose oxidation by tissue. This phenomenon was shown by Tepperman (7) with an observed increased RQ in rats fed for one hour a day a diet that contained a high proportion of carbohydrate, sucrose, relative to that of a standard laboratory chow diet. Alternatively, a high-fat, low-carbohydrate diet suppresses lipogenesis in the liver (50-54) and in adipose tissue (38, 55, 56) of ad libitum -fed animals. Tepperman showed that intermittent feeding of a high-fat diet did not enhance intestinal glucose absorption, liver hexokinase activity, nor glucose utilization when compared with ad libitum-fed controls. It is important to assess the extent to which metabolic adaptation with altered meal pattern is influenced by composition of the diet, or to what extent adaptive changes occur due only to the presence of a large supply of certain substrate. The composition of the diet, particularly the concentration and form of carbohydrate, is probably the most significant factor in regard to an observed response to meal-feeding relative to a nibbling diet.

In order to demonstrate the influence of diet composition, Leveille (23) performed experiments using diets with dietary fat supplied at levels of 10, 20, and 30 percent of the diet, corresponding with 21, 38, and 52 percent of calories derived from fat. Given adjustment of the protein component of the diet to maintain an essentially constant protein to calorie ratio, Leveille was able to discern that previously observed physiologic differences between tissue of meal-fed and nibbling rats were no longer apparent when the diets contained large amounts of fat. Similar results were observed by Vaughan and Winders (57) in that high-fat diets markedly inhibit the activity of pentose pathway enzymes, namely glucose-6-phosphate dehydrogenase and NADP-malate dehydrogenase in livers of rats fed for two hours daily.

Effect of increased level of dietary protein concurrent with decreased levels of carbohydrate served to diminish rates of fatty acid synthesis (51). Similar differences were apparent at all levels of dietary protein fed to meal-fed rats and nibbling rats. In both cases, malic enzyme activity decreased as dietary protein increased. Whether observed effects of protein feeding could be due to the reduction of dietary carbohydrate was not known. However, study of the influence of dietary protein and fat level on hepatic lipogenesis in the chicken suggests that dietary protein and fat exert a direct effect on the capacity for fatty acid synthesis which can not totally be accounted for by the reduction in dietary carbohydrate (58). It appears that meal composition may influence the rate for lipogenesis to proceed, but may not provide a total answer to questions posed by Baker's observed results.

The experimental animal--A touchstone element.

It is necessary to emphasize that interpretation of meal frequency investigations is dependent upon the particular animal species used in the study. In light of the relatively slight amount of information available in human research, this influence of the particular animal species used becomes of significant concern. Mechanics of the physiological system vary between species and sometimes even within animal strains, and pathways may be sufficiently dissimilar that the effect of meal pattern alteration in one species may be different from that in another unrelated species. Traditionally, laboratory models include the laboratory rat, the swine and the chicken.

Variation in response to altered meal frequency may be anticipated between species, i.e. the pig compared to the laboratory rat; but that strain differences exist even within rat species has been elucidated. BHE and Wistar rats were compared as either meal-fed or ad libitum-fed models (59). Meal-fed BHE rats had greater levels of carcass lipid than did meal-fed Wistar rats. Liver lipids were affected by strain in ad libitum-fed rats, and carcass

cholesterol levels also varied between strains similarly treated. Thus the problem with interpretation of numerous animal studies in respect to the particular physiologic make-up of man is complex.

A. The pig as the animal model: Swine have been used in meal frequency research and have found favor among some investigators with their apparent physiologic similarities to man (60). Researchers have found that pigs readily adapt to either type of feeding pattern, but that research with swine reveal conflicting results in response to meal-feeding. Some experiments have achieved greater weight increments as a result of less frequent feedings of two meals daily versus feeding four times a day (61, 62), while some indicate that groups fed ad libitum maintained tendency for greater body fat formation than did animals fed twice (63) or three times daily (64).

Despite the above reports, research does not generally support the notion that total metabolism or carcass quality responds to meal-feeding (65, 66). Activities of the three dehydrogenase enzymes active in the pentose pathway and for NADPH generation were assayed in adipose tissue samples, and at no time period were values for the two groups, meal-fed or nibbling controls, significantly different (67). O'Hea and Leveille (68) concluded that the primary lipogenic tissue in the pig was adipose with its high in vitro synthetic capacity. Why adipose tissue in the pig does not respond in a similar manner to that of the rat when subjected to meal-feeding is uncertain. Differences may be attributable to fundamental variation between adipose tissue of the two species; e.g. pig adipose in vitro fatty acid synthesis is relatively insensitive to added insulin relative to that of rat tissue (67). Other physiological differences between the species exist, including some that may not yet be known to man, but that would further contribute to conflicting research reports.

A more recent report by the team of Allee, Romsos, Leveille and Baker (69) indicated that a hyperlipogenic state could be induced in pig adipose by meal ingestion which compared with the superior lipogenic capacity shown for meal-fed rats. Authors indicated that previous work, done in their own laboratory, had been conducted using older and larger pigs (67) and may have contributed towards contradicting results. Meal-eating failed to produce adaptive changes in lipogenic capacity in the earlier report, presumedly because a postabsorptive state was not reached between meals. Increased enzyme activity related to fatty acids synthesis was also recorded for meal-fed pigs (69) corresponding more closely with results using rats as the animal model than had previously been indicated.

Anderson and Fausch (70) investigated influence of feeding frequency on serum lipid levels. Animals fed ad libitum were compared to those fed one or two times daily. Results were identical after 84 hours and following 173 days of experimental feeding. Animals with free access to food had higher cholesterol but lower triglyceride levels than did animals fed 2 hours daily. In this respect, response to meal-feeding for the pig differs relative to Leveille's (67) observations and that of Cohn (71) in their investigations with the chicken used as the animal model. Okey et al. (72), using female rats and Wells et al. (73), using rabbits showed similar response to those observed by Leveille (74) who evaluated response in chickens with respect to serum cholesterol levels. Indeed, the complexities of determining an appropriate animal model are plainly apparent.

B. The chicken as animal model: Meal-eating in the chicken, another monogastric animal, has been less extensively investigated but information available suggests significant biological differences when compared to the rat. Adaptive response of the digestive tract to altered feeding frequency in fowl has been described by Lepkovsky and colleagues (75). After four to six

weeks of experimentation comparing chicks trained to a gorging diet whose food was available only two hours a day with those fed in the usual manner, it was observed that crops of trained animals were markedly increased in size but neither the stomach, gut nor pancreas was affected. Proteolytic activity in the pancreas and gut, glycogen synthesis and lipogenesis in both liver and adipose tissue were of equal value or even reduced in trained meal-eaters. Authors concluded that adaptive changes in chicks trained to ingest "large spells of food" is confined to hypertrophy of the crop, and thus enables them to store large amounts of food.

C. Inconsistencies exist between species. Metabolic differences between the chick and the rat have been indicated (76) since meal-fed chicks deposited less body fat than did ad libitum-fed controls. More recently Leveille and Hanson (74) showed similarities of the meal-fed fowl with the intermittently starved rat. Researchers observed gastric hypertrophy with increased weight of the digestive tract relative to body weight for the meal-eating versus the nibbling chick. However, unlike the rat metabolism, rate of lipogenesis remained unchanged in meal-fed chicks compared with ad libitum-fed controls (75). O'Hea and Leveille (68) determined that isolated chick adipose tissue had a low capacity for fatty acid synthesis. The majority of de nova fatty acid synthesis in the chick occurred in the liver with adipose tissue appearing to function primarily in lipid storage (77). This phenomenon is in contrast with the rat metabolism wherein the liver is secondary for fatty acid biogenesis and adipose assumes the primary role. Furthermore, liver lipids and liver cholesterol levels were not significantly altered for the chick by meal-eating. Nonetheless, results described by Leveille and Hanson (74), and supported by Feigenbaum and others (76), propose that the chick and rat may metabolically respond to intermittent food intake in basically an analogous fashion.

Feeding frequency and body fat.

Question remains regarding meal frequency and its ability to affect altered body composition. Recently, several researchers have found evidence to claim results that contraindicate those of previous years. It had earlier been reported that meal-fed animals, including those either force-fed (11,78), fed for one or two hours per day (79), or fed with periodic alternation of two-day fasts and five-day periods of repletion (80) showed an increased percentage of total carcass lipids compared with ad libitum-fed controls. Tepperman and co-workers reported these results first in the 1940's (7) and again in the 1950's (25, 80). Fabry confirmed these results in the majority of his experiments, indicating that intermittently fasted rats or those fed for two hours a day had higher proportions of total body fat than did controls (81, 82). Welch (83) found that intermittently starved mice had higher percentages of body ether extract indicating increased deposition of fat, but that feeding schedule had no appreciable effect on body weight. Body fat increases were not indicated when food was withheld for only a single 24 hour period per week, and thus the author deduced that the single-feeding time schedule was only marginal in providing stimulus for enzyme adaptations to occur. However, once the mechanism had been altered by meal- or force-feeding, the condition persisted even when the animal was returned to its natural feeding habits.

It would appear that the meal-fed animal, more specifically, the force-fed animal, is more efficient in converting ingested calories to stored energy in the form of body fat. Furthermore, the mechanism enabling the adaptation to occur appears to persist even after the animal is allowed to resume his natural eating behavior. Muiruri and Leveille (84) noted that lipogenic enzyme activity in adipose tissue of converted rats, i.e. meal- eaters returned to a nibbling diet, decreased only slowly relative to that of control nibblers. Hollifield et al. (35) and DeBont (85) supported the results of

Muiruri and Leveille. Observations reported in converted rats may be supported by Heggeness (86) who noted that rats fed a restricted maintenance diet retained a significantly greater fraction of ingested calories than did ad libitum-fed individuals.

However in recent years, there have been several reports from the laboratory of Romsos and Leveille (30, 87-90) that lend evidence to contradict the theory that the effect of less frequent food intake is manifest by a greater accumulation of body fat. A four-week study with mice fasted for two separate 24 hour periods per week failed to show change in body weight or fat content; however, alterations of in vitro lipogenic capacity of liver and adipose tissue were apparent. It thus appears that three parameters exist that must be considered before interpreting results from meal frequency research: (a) the amount of caloric restriction in the diet, (b) duration of study, and (c) the initial adaptation period.

A. Caloric restriction of the diet: Several studies exist that support relationship of severity of caloric deprivation with subsequent influence of meal frequency. Mice intermittently-fed frequently have decreased food consumption relative to ad libitum intake levels (79). Findings of Hoffman and others (91) showed that a group of meal-fed rats whose caloric intake was restricted relative to ad libitum-fed controls exhibited higher percentage of lipid and a lower percentage of water in tissues for rats of all ages. Thus the group weighed less but was more obese than controls. A higher ratio of fat in intermediately fasted rats was observed by Fábry et al (81) even with their considerably reduced caloric intake, compared with animals consuming diets divided into small portions and fed throughout the day. Cohn and Joseph (92) found that carcasses of force-fed rats contained increased amounts of body fat and less protein than ad libitum eating partners even when animals were pair-fed. When diets were restricted to approximately 33 to 40 percent of "normal" intake, periodicity of food intake failed to influence either the amount of weight loss or body composition. Results thus suggest that a "threshold" for caloric intake exists that must be exceeded in order that changes in body composition resulting from alteration in meal frequency may be observed, and that differences fail to be manifest if diets of animals fed in either manner are subsequently reduced in calories.

B. Duration of the experiment: Variable duration of experimental feeding has reportedly influenced research results. Cohn and Joseph (12) compared ad libitum-fed rats with animals pair-force-fed for a period of two weeks. They determined that force-feeding resulted in a 13.2 percent increase in body fat relative to a 4.5 percent increase for rats on an ad libitum-feeding schedule. Similar results have been indicated in other short-term feeding studies (23,93). In contrast, DeBont and co-workers (85) found that meal-fed rats contained similar percentages of body fat as nibbling controls, but only after 12 weeks of experimental feeding. Results were 16 percent compared to 17 percent body fat, respectively. Leveille (94) had previously indicated that the observed increased energy efficiency may dissipate with longer term studies and several months of meal-feeding. In the more recent study reported by DeBont et al. (85), rats switched from meal-feeding to an ad libitum-feeding pattern failed to exhibit an immediate return of the in vitro rate of fatty acid synthesis to values for nibbling rats. A period of not less than six weeks was required for the lipogenic rate to return to the nibbling level. When converted rats were returned to meal-feeding, reversal of the fatty acid synthetic rate was markedly more rapid. Previously attained high levels were observed within ten days of meal-eating. This apparent languor with readaptation from meal-eating to nibbling diets had been reported by others in earlier studies (84, 95).

C. The initial adaptation period: Reports from recent investigations of Romsos' research team (85, 89, 90) have alluded to an apparent gross

misinterpretation of early research. Initial adaptation period of animals to their respective diet had been largely ignored, and Romsos and co-workers have reportedly been able to document the consequence of this adaptation period on research results. This factor is an inherent contributor in studies where the effect of switching diets for experimental subjects is examined and may include a residual latent response of lipogenic rate. DeBont et al. (85) demonstrated the profound influence of an initial adaptation period when comparing meal-fed and nibbling rats. During week one of a 12 week study of meal frequency, meal-fed rats failed to gain weight. After adaptation to meal-eating, they gained at about identical levels to that of nibbling animals until the sixth week of the study. It has been recognized that growth rates of animals and children may be retarded by food restriction during early stages of development. Catch-up growth, or compensatory growth, has been attributed to the replacement of body fat depleted during restriction (96, 97). Ozelci, with Romsos and Leveille (89), found that pair-feeding force-fed and ad libitum-fed rats without an initial adaptation period failed to manifest differences in weight gain or body fat between the two groups. Ozelci (90) went on to effectively eliminate the effect of the relatively increased food intake of controls by pair-feeding rats during a refeeding program following food restriction. Restricted-refed rats gained more body weight than did control animals. Compensation was apparent within the first week of refeeding and continued for two weeks following in rats who had previously been fed diets restricted to 50 percent or less of ad libitum food intake. Catch-up growth was most marked in severely restricted animals.

Recent evidence exists that supports DeBont and Ozelci's postulate that it is actually the "shift" from one level of meal frequency to a higher level that contributes to the observed metabolic adaptation and enhanced food efficiency in rats (85, 89). It is not clear at this time what forces exist that come into play and affect the energetics of caloric conservation, but that basal metabolic rate (BMR) is decreased as a result of restricted food intake has been suggested (98-100). If BMR does not increase immediately when food intake is augmented, then the increased and unused increment of energy consumed is available for fat deposition. Sharma et al. (101) in 1973 with young pigs, and even more recently in 1980, Schoenborne and Canolty (102) with rats have failed to substantiate differences in net energy values, nor in final body fat and lean composition of animals following the two types of dietary regimes. Feed efficiency was not enhanced with increased frequency of food intake. Therefore, extrapolation of laboratory results with a variety of research animals may not provide answers to questions regarding meal frequency in the human; but they do serve to suggest hypotheses that may be useful to man.

Role of altered meal frequency in weight control for man.

The role of meal frequency and distribution of energy intake in a weight reduction program has implication in control of human obesity. Obese humans who consume a restricted diet in order to force weight reduction, followed by a rapid return to the previous level of energy intake with weight reduction, is analogous to our restricted-refed animal. Compensatory fat gain with a return to normal eating habits is a definite psychological deterrent for achieving permanent weight loss.

Increased fat cell size or number as causative agent for body fatness?

Two factors, increased number or increased size of adipocyte cells are identified for gain of body fatness. Attention has been given to the "fat cell theory" whereby an increased number of fat cells in adipose tissue predisposes animals, including man, to obesity (103). Research with animals by Hirsch and colleagues (104, 105) has shown that overfeeding rats in the first few weeks of life could lead to permanent obesity accompanied by an

increase in number of fat cells in adipose tissue. It was believed that later nutritional manipulation could affect adipose tissue mass by changes in fat cell size but not cell number; yet Braun and co-workers (106), also in rats, had documented an increased fat cell number in adipose tissue with meal-feeding and intermittent fasting.

There has been little investigation relating directly to meal-feeding and fat deposition in increased cell size or number. Romsos & Leveille (107) showed slightly decreased fat cell size for meal-eaters relative to nibbling animals with no difference in fat cell number. Results, however, were attributed to significantly reduced body weight for meal-eating animals, indicating that results of earlier investigation may have been erroneously interpreted.

Research with human subjects is lacking in this area and more research is required before substantive conclusions may be drawn in relation to fat cell size or number and tendency for obesity in man.

Meal frequency and the insulin response.

Hormonal secretions in animals accustomed to consuming large amounts of food in a short time period appear distinctly different relative to hormonal patterns in nibbling animals. Three factors exist that may alter hormonal secretion and affect metabolism. First, increased blood concentration of an absorbed nutrient can stimulate output of a hormone, e.g. insulin is secreted as result of elevated glucose concentration in blood. Periodic loads of carbohydrate with intermittent feeding and resulting hyperinsulinemia may lead to functional exhaustion of the beta-cells. Mosinger et al. (108) found a decreased insulin content and increased granulation of the beta-cells of the pancreas in rats subjected to such a meal regimen. Second, the hormone may play a permissive role with the actual stimulus for alteration being a dietary component. It may be required, however, that a minimum amount of hormone be present in order that adaptive changes take place. Third, the diet may induce tissues to become more sensitive to a particular hormone, and thus affect a hormone-dependent metabolic change (93). Regardless of the particular mode of action, hormonal involvement in metabolic adaptation to meal-eating is indicated.

Researchers have concluded that the insulin response in rats may be analogous to the situation characterizing the diabetic state in man (109). Pair-force-fed animals were less sensitive to insulin and showed a delay in glucose clearance from intravenous blood relative to ad libitum-fed controls. These findings are consistent with those of Gwinup et al. (110), Fabry et al. (111), and Nunes et al. (112) who reported that a decreased frequency of food intake is accompanied by diabetic-type glucose tolerances. By contrast, Leveille (28) and Braun (113) used force-fed but undernourished animals and showed an increased sensitivity to insulin. Such animals were able to dispose of blood glucose at an accelerated rate relative to their ad libitum - eating counterparts.

In a study using human subjects, Schierf and Raetzer (114) showed that individual insulin peaks were twice as great with a three-meal per day regimen as when six meals were consumed. Analysis of insulin profiles showed individual areas under peaks for 24 hour periods. The conclusion was drawn that plasma insulin response was a function of the load of calories requiring deposition and did not appear to depend on the frequency with which the calories were ingested. The greatest demand for insulin is with the meal-eater, since it may be assumed that individuals consuming fewer meals per day have more calories per meal than would one who spaced meals throughout the day. The possibility for inducing onset of diabetes with meal-eating may be enhanced for those predisposed to the disease since it is the demand for

insulin at a given time that creates the potential diabetic condition.

Houssay and Martinez (115) suggested that early onset of diabetes may be attributable to any or all of three factors: (a) an increased hyperglycemic reaction following the single daily feeding regimen resulting in greater load on pancreatic islet cells. Eventually the cells may become exhausted and diabetes is initiated, or (b) greater fat content of animals fed once daily results in increased tendency towards obesity and propensity for the disease, or finally (c) enhanced enzymatic stimulus for synthesis of insulin as a result of an infrequent feeding pattern leads to relative insulin deficiency. Any or all of these factors may contribute toward development of diabetes. All interpretations appear plausible in view of the metabolic necessity the meal-eater imposes on body systems for disposing with large amounts of calories and nutrients which flood the body of the "gormandizing" individual.

Meal frequency and cardiovascular disease. Is there a connection? Research data that supports an enhanced rate of lipogenesis with meal-eating has led to increased attention being directed toward a presumed relationship of meal frequency on serum cholesterol levels and subsequent development of atherosclerosis. It is beyond the scope of this review to deal with the controversy of diet in relation to incidence of coronary heart disease since no consensus exists on this issue even among salient professionals. However, high serum cholesterol has been associated with atherosclerosis and altered meal frequency has been shown to affect serum cholesterol. Cohn et al. (116) fed chicks various diets containing 4 to 5 percent cholesterol. Diets were consumed either ad libitum or were made available for 2 hours daily. After five weeks of feeding, the incidence of atherosclerotic lesions was several times higher in meal-fed chicks as opposed to those allowed free access to food. Subsequently, authors followed the 3 to 4 percent cholesterol diets with atherogenic diets of 1 percent cholesterol fed ad libitum, and then sequenced this with a cholesterol-free diet. Similar results were reported with more rapid regression of pathological changes observed for animals allowed free access to food than for those who continued to be meal-fed. In two later studies, Okey et. al. (117) reported that although feeding pattern did affect physical composition of both plasma and liver lipids in rats, the total amounts of serum and liver cholesterol, triglyceride and phospholipid were not appreciably altered by feeding method. In support of work done earlier by Cohn (109), Anderson and Fausch (70) reported in 1964 that serum cholesterol and phospholipid levels in ad libitum-fed pigs were significantly higher than in restricted groups fed for either 1 or 2 hours daily. Most recent investigation by Romsos et al. (118) in 1978, and others (26, 119) support the opposite, that plasma cholesterol and triglyceride levels were not altered by feeding schedule. One study had used humans as subjects (26), one had used pigs (118) and another had used the dog as the experimental subject (119). Therefore, in his reports, Romsos postulated that meal frequency generally appears to have minimal influence on circulating cholesterol levels.

Research with primates as the animal model has been conducted and historically correlates well with investigation using human subjects. Influence on cholesterolaemia in monkeys was first demonstrated by Gopalan et al. (120) in 1962. Gopalan found that meal-feeding monkeys induced greater body weights than for controls who ate at four undefined intervals. The restricted group experienced much higher serum cholesterol levels during the eight - week feeding trial. A later report by Jagannathan and Gopalan (121) reported concurring results, and monkeys, meal-fed a high fat diet once daily, exhibited elevated serum cholesterol levels relative to those eating the same diet provided in two portions.

Apparently, the ability to affect changes in serum cholesterol levels relative to the postulated role as an atherogenic stimulus depends greatly upon the particular species, sex, age and diet composition of the experimental subject. For instance, sex appears to have a pronounced effect on serum lipid levels in swine with intact males having consistently lower values than either the females or castrated males (70). Human serum cholesterol levels are generally lower for the premenopausal female than for the male, although the reverse is true for the female rat (122) and rabbit (123). Thus, generalities should not be concluded.

Meal frequency in man has primarily focused on the metabolism of plasma lipids, more particularly cholesterol and development of cardiovascular disease. Fabry and colleagues (124) studied 1359 men ages 60-64 years and found relationship with meal frequency and occurence of ischaemic heart disease. In a comprehensive study of the effect of size and frequency of feeding upon metabolism, Irwin and Feeley (125) served a three-meal regimen with half the daily calories consumed at the evening meal, and compared this with feeding both three or six equal-sized meals. No significant differences were indicated for nitrogen retention, fat digestibility nor for serum lipid levels of phosphoplipids, glycerides and total fatty acids. Difference was noted for serum cholesterol level and a lower level was attained when three equal-sized meals were fed than when two small and one large meal per day were consumed. Other researchers have presented data that corroborate these results. Cohn (126) reported that he was able to correct pathogenesis in a patient with ideopathic hyperlipidemia and cholesterolaemia with a change in meal habits that included frequent meals of small portion size. Gwinup et al. (110, 127) have reported similar results and observed that hospital patients consuming a single daily meal maintained elevated serum cholesterol levels concurrent with a lowered glucose tolerance than did those whose meal was proportioned into ten smaller servings.

In summary of the relationship to cardiovascular disease, indication is thus that feeding frequency has some effect on plasma cholesterol levels in man and may be related to pathogenesis of atherosclerosis and coronary heart disease. However, by no means should studies reported to date be considered conclusive. Shortcomings predominate in human research experiments which may be attributable to the feasability of studying only small numbers of subjects and lack of control over the amounts and composition of diets being consumed. Cognizant evidence exists that diseases including obesity, diabetes and coronary heart disease plague our national health. Overweight, reduced glucose tolerance and hypercholesterolaemia, all of which are risk factors for ischaemic heart disease, are more common in elderly subjects with an infrequent meal pattern than in those consuming a large number of smaller meals (111). Periodic loading of food consumed during infrequent meals may more readily promote deposition of fat reserves, damage beta-cells of the pancreas (thus predisposing the individual toward diabetes), or affect increased circulating levels of serum cholesterol.

Will increased meal frequency by helpful in successful weight loss programs?

In light of result of research using human subjects, it is curious when one considers altered habits assumed by individuals in efforts to lose weight. Oftimes, the first constraint of a "diet" is to reduce the number of meals eaten each day. For instance, breakfast may be omitted in an effort to reduce total daily caloric intake. The majority of food intake is then

diverted to the evening period, and researchers have shown that if indeed results with meal-fed laboratory animals may be extrapolated to man, the situation imposed is not one that tends to promote weight loss.

Some researchers have reported that meal-fed animals have lower levels of activity particularly during evening hours than their nibbling counterparts. Leveille (128) was unable to prove that reduction occurred in either basal metabolism rate or heat production, and he concluded that greater feed efficiency of the meal-fed rat was the result of a reduced activity level and thereby reduced energy expenditure. Obese humans may, in general, ingest fewer calories than non-obese individuals (129, 130), but reduced activity of the obese has also been demonstrated and may serve to explain the apparent paradox. Among the first studies to compare activity levels of obese and non-obese individuals was reported by Johnson, Burke and Mayer (131) in 1956. Diet, physical maturation and activity of 28 obese and non-obese girls of similar height, age and school grade were investigated. Average daily caloric intake of the obese group of girls was, in most cases, lower than for the non-obese. However, non-obese girls spent more hours each week in energy-demanding activities. Similar, yet less dramatic differences were observed in adolescent boys (132). Motion pictures used to compare activity levels of obese and non-obese girls in a summer camp again supported that obese girls were far less active than their lean counterparts (133). In addition, Garne (134) has related that both obese and lean girls frequently indicated owning bicycles, but that the obese were more frequently observed standing beside them. Thus, it seems that a more logical recommendation in weight reduction programs is not so much to decrease meal frequency but to increase the level of physical activity with a concurrent reduction in total calories consumed.

The philosophy with the dieting-obese individual is that by eating less frequently, daily caloric intake will naturally be reduced. This may be true for untrained rats but does not seem valid for humans. A Northcentral regional study on preschool children (135) showed that food energy intake was inversely proportional to number of meals consumed in children who ate less than four meals per day; yet, only 19 percent of children reporting low intakes ate six or more meals per day. Leon (136) in 1977 showed that of patients who lost weight, the ones who regained the weight had higher frequency of between-meal food consumption in addition to eating lunch in the afternoon. The lack of effectiveness in achieving weight reduction with simple alteration of meal pattern has been recorded by several researchers. Their reports indicate that frequency of feeding in man was not found to effect skinfold thickness (6), body weight nor body composition (5, 112, 137, 138).

Hence it is apparent that the efficacy of employing altered meal patterns as part of a weight reduction program is dubious indeed. Research results are conflicting and altogether inconclusive. In order to discuss successful weight loss, it is necessary to consider "caloric balance" throughout the day and avoid emphasis on "meal balance". Pounds are lost by ingesting fewer calories than are expended and not by manipulating frequency distribution of a superfluous caloric intake.

Meal frequency and "our snacking existence." Should snacking be condemned or condoned?

The effect of meal frequency on metabolic processes is pivotal in discussion of the controversy with America's confirmed snacking existence. Snacking has become an American way of life and snack foods are frequently consumed between meals. Snack foods, if properly chosen, serve to compliment the nutrient intake and may fulfill a nutritional void that would otherwise exist in an individual's diet. This is particularly true with regard to children and teenagers (139, 140). Caution must be practiced since a high proportionate consumption of these foods may reduce nutrient variety furnished by the basic four food groups and excesses may easily contribute to the onset of obesity.

Individual choice regarding frequency of food intake remains more a psychosocial preference than a health-related issue. In a confidential written questionnaire distributed by Young (26), respondents described feelings in terms of preference for meal frequency. Of men fed a single daily meal, several commented on an overwhelming desire and need for sleep in the two-hour period immediately following the meal. In addition, extreme hunger experienced during the day with the one-meal regimen was distracting for subjects. When offered their choice between eating one or six meals per day, 40 percent preferred the single meal for reasons related to convenience and economy of time; others enjoyed the greater taste satisfaction and social relaxation surrounding the occasion of the single meal. Thirty percent preferred eating six meals daily and the remaining 30 percent were ambivalent. It seemed physically easier to consume six meals daily, but subjects commented that food seemed to dominate their life when meals were so frequently consumed. However, efforts to restrict caloric intake may dictate reduced frequency of food intake, since it may simply take more willpower than some persons possess to say "No" to appetite ten times each day (141).

We may not categorize the obese as "meal-eaters" and their leaner counterparts as "nibblers", or vice versa. Fabry mentioned that an elderly man he observed in a study was 64 percent overweight and could be classed as a nibbler (111). The individual confessed that he ate practically all the time and "enjoyed his food intake extremely." His condition of overweight was completely in harmony with his fancy. The thinnest man in Fabry's study was not a nibbler, but a meal-eater. He was divorced, a taxi driver and a man who apparently not only took his meals very irregularly but also ate very little. Conversely, we return to the scene with Stare's "slender beauties of Bali" -- eating constantly but never becoming fat. What do these results suggest? -- simply that meal frequency has consequence in determining metabolic activity, but that total caloric intake is obviously the primary limiting factor in reference to genesis of obesity and possibly other pathologic diseases. Food habits considered desirable include that one meet his/her nutritional requirements while maintaining an ideal weight. Today, technology has provided man with a readily available, relatively consistent food supply and meal frequency is dictated primarily by personal preference -- a choice that is often more reflective of psychological than of biological perceptions of need.

REFERENCES

1. HERMANN Newspaper article from National Observer of Nov. 16, 1964.

2. COHN, C. Feeding patterns and some aspects of cholesterol metabolism. Fed. Proc. 23:76-81, 1964.

3. HOLECKOVÁ, E. Hyperphagia and gastric hypertrophy in wild Norway rats. Physiologia bohemoslov. 11: 465-469, 1962.

4. FÁBRY, P. Metabolic consequences of the pattern of food intake. In Code, C.F. (ed). Handbook of Physiology. Washington, D.C.: Am. Physiol. Soc., 1967.

5. YOUNG, C.M., SCANLAN, S.S., TOPPING, C.M., SIMKO, V. and LUTUAK L. Frequency of feeding, weight reduction and body composition. J. Am. Dietet. A. 59:466-472, 1971.

6. THOMAS, J.A. and CALL, D. Eating between meals--a nutrition problem among teenagers? Nutr. Rev. 31:137-139, 1973.

7. TEPPERMAN, J., BROBECK J.R. and LONG, C.N. H. The effects of hypothalmic hypophagia and of alterations in feeding habits on the metabolism of the albino rat. Yale J. Biol. Med. 15:855-874, 1943.

8. HOLECKOVÁ, E. and FÁBRY, P. Hyperphagia and gastric hypertrophy in rats adapted to intermittent starvation. Brit. J. Nutr. 13:260-266, 1959.

9. FÁBRY, P. and TEPPERMAN, P. Meal Frequency--a possible factor in human pathology. Am. J. Clin. Nutr. 23:1059-1068, 1970.

10. LEVEILLE, G.A. Adipose tissue metabolism. Influence of periodicity of eating and diet composition. Fed. Proc. 29:1294-1301, 1970.

11. COHN, C. and JOSEPH, D. Changes in body composition attendant on force feeding. Am. J. Physiol. 196:965-968, 1959.

12. COHN, C. and JOSEPH, D. Role of rate of ingestion of diet on regulation of intermediary metabolism ('meal-eating' vs. 'nibbling'). Metab. Clinc. Exptl. 9:492-500, 1960.

13. JACKSON, C.M. Effect of acute and chronic inanition upon the relative weights of the various organs and systems of adult albino rats. Am. J. Anat. 18:75-111, 1915.

14. FÁBRY, P. and KUJALOVÁ, V. Wachstum des Dunndarmes bei intermittierend hungerndee Ratten. Naturwissenschaften. 45:373-374, 1958.

15. LOJDA, Z. and FÁBRY, P. Histologie and Histochemie der an intermittierends Hungern adaptierten Pattan. Acta Histochem. 8:289-300, 1959.

16. POCKNEE, R.C. and HEATON, W.F. The effect of feeding frequency on the growth and composition of individual organs in the rat. Brit. J. Nutr. 35:97-104, 1976.

17. STEVENSON, N.R. and FIERSTEIN, J.S. Circadian rhythms of intestinal sucrose and glucose transport: cued by time of feeding. Am. J. Physiol. 230:731-735, 1976.

18. KUJALOVÁ, V. and FÁBRY, P. Intestinal absorption of glucose, fat and amino acids in rats adapted to intermittent starvation. Physiologia bohemoslav. 9:35-41, 1960.

19. CHAKRABARTY, K. and LEVEILLE, G.A. Influence of periodicity of eating on the activity of various enzymes in the adipose tissue, liver and muscle of the rat. J. Nutr. 96:76-82, 1968.

20. LARRALDE, J. Effect of length of fast upon the absorption of glucose from the intestine of rats. Revta esp. Fisiol. 3:31-39, 1947.

21. MAGEE, H.E. The physiology and treatment of starvation. Starvation and protein hydrolysates. Proc. R. Soc. Med. 38:388-398, 1945.

22. FÁBRY, P. Feeding Patterns and Nutrition Adaptations. Prague: Academia, 1969.

23. LEVEILLE, G.A. Adipose tissue metabolism: influence of periodicity of eating and diet composition. Fed. Proc. 29:1294-1307, 1970.

24. FABRY, P. Handbook of Physiology. Alimentary Canal. Washington, D.C.: Am. Physiol. Soc., 1967.

25. TEPPERMAN, J. and TEPPERMAN, H.M. Effects of antecedent food intake on hepatic lipogenesis. Am. J. Physiol. 193:55-64, 1958.

26. YOUNG, C.M., HUTTER, L.F., SCANLAN, S.S., RAND, C.E., LATWAK, L. and SIMKO, V.J. Metabolic effect of meal frequency on normal young men. J. Am. Dietet. A. 61:391-398, 1972.

27. WADHWA, P.S., YOUNG, E.A., SCHMIDT, K., ELSON, C.E., and PRINGLE, D.J. Metabolic consequences of feeding frequency in man. Am. J. Clin. Nutr. 26:823-830, 1973.

28. LEVEILLE, G.A. and CHAKRABARTY, K. Absorption and utilization of glucose by meal-fed and nibbling rats. J. Nutr. 96:69-75, 1968.

29. WILEY, J.H. and LEVEILLE, G.A. Significance of insulin in the metabolic adaptation of rats to meal ingestion. J. Nutr. 100:1073-1080, 1970.

30. ROMSOS, D.R. and LEVEILLE, G.A. Effect of meal frequency and diet composition on glucose tolerance in the rat. J. Nutr. 104:1503-1512, 1974.

31. LEVEILLE, G.A. and CHAKRABARTY, K. Diurnal variations in tissue glycogen and liver weights of meal-fed rats. J. Nutr. 93:546-554, 1967.

32. JANSEN, G.R., HUTCHISON, C.F., and ZABETTI, M.E. Effect of dietary fat and starvation on conversion of C^{14} glucose into fat and turnover of newly synthesized fat. Biochem. J. 99:323-332, 1966.

33. LEVEILLE, G.A. In vivo fatty acid synthesis in adipose tissue and liver of meal-fed rats. Proc. Soc. Exp. Biol. Med. 125:85-88, 1967.

34. TEPPERMAN, J. and TEPPERMAN, H.M. Adaptive hyperlipogenesis--late 1964 model. Ann. N.Y. Acad. Sci. 131:404-411, 1965.

35. HOLLIFIELD, G. and PARSON, R.W. Metabolic adaptation to stuff and starve feeding program. I. Studies of adipose tissue and liver glycogen in rats limited to short daily feeding periods. J. Clin. Invest. 41:250-253, 1962.

36. ROMSOS, D.R. and LEVEILLE, G.A. Influence of periodicity of eating on intestinal glycolytic enzymes in the rat. Comp. Biochem. Physiol. 49A:533-540, 1974.

37. HAHN, P. and SKALA, J. The development of some enzyme activities in the gut of the rat. Biol. Neonate 18:433-438, 1971.

38. LEVEILLE, G.A. and HANSON, R.W. Adaptive changes in enzyme activity and metabolic pathways in adipose tissue from meal-fed rats. J. Lipid Res. 7:46-55, 1966.

39. LEVEILLE, G.A. and HANSON, R.W. Influence of periodicity of eating on adipose tissue metabolism in the rat. Can. J. Physiol. Pharmacol. 43:857-868, 1965.

40. HOLLIFIELD, G. and PARSON, R.W. Effect of feeding on fatty acid synthesis. In: Handbook of Physiology. Adipose tissue. Washington, D.C.: Amer. Physiol. Soc., 1965.

41. LEVEILLE, G.A. Control of lipogenesis in adipose tissue of fasted and fed meal-eating rats. J. Nutr. 92:460-466, 1967.

42. TEPPERMAN, H.M. and TEPPERMAN, J.: Patterns of dietary and hormonal induction of certain NADP-linked liver enzymes. Am. J. Physiol. 206:357-361, 1967.

43. TEPPERMAN, J. and TEPPERMAN, H.M. Metabolism of glucose-1-C^{14} and gluc-6-C^{14} by liver slices of refed rats. Am. J. Physiol. 200:1069-1076, 1961.

44. FOSTER, D.W. and SRERE, D.A. Citrate cleavage enzyme and fatty acid synthesis. J. Biol. Chem. 243:1926-1930, 1968.

45. FITCH, W.M. and CHAIKOFF, C.I. Enzyme activities in liver. J. Biol. Chem. 235:554-557, 1960.

46. FRIEDLAND, R.A. and HARPER, A.E. Metabolic adaptations in higher animals. J. Biol. Chem. 234:1350-1354, 1959.

47. TEPPERMAN, H.M. and TEPPERMAN, J. 'Adaptive' hyperlipogenesis. Fed. Proc. 23:73-75, 1964.

48. KIMURA, T., MAJI, T. and ASHIDA, K. Periodicity of food intake and lipogenesis in rats subjected to two different feeding plans. J. Nutr. 100:691-697, 1970.

49. BAKER, N. and HUEBUTTER, R.J. Rapid activation and inactivation of fatty acid synthesis from glucose in vivo. J. Lipid Res. 13:329-337, 1972.

50. BAKER, N., PALMQUIST, D.L. and LEARN, D.B. Equally rapid activation of lipogenesis in nibbling and gorging mice. J. Lipid Res. 17:527-535, 1976.

51. LEVEILLE, G.A. Effect of periodicity of eating and diet composition on enzymatic adaptations. Nutr. Proc. 9th Int. Cong. Nutr. Mexico, 1972. 1:13, 1975.

52. HILL, R., LINAZASORA, J.M., CHEVALIER, F. and CHAIKOFF, I.L. Regulation of hepatic lipogenesis: The influence of dietary fats. J. Biol. Chem. 233:305-310, 1958.

53. HILL, R., WEBSTER, W.W., LINAZASORA, J.M. and CHAIKOFF, I.L. Changes in lipid synthesis after fat feeding. J. Lipid Res. 1: 150-153, 1960.

54. WHITNEY, J.E., ROBERTS, S. Influence of previous diet on hepatic glycogenesis and lipogenesis. Am. J. Physiol. 181:446-451, 1955.

55. HAUSBERGER, F.X. and MILSTEIN, S.W. Dietary effects on lipogenesis in adipose tissue. J. Biol. Chem. 214:483-488, 1955.

56. WOOD, J.D. and REID, J.T. The influence of dietary fat on fat metabolism and body fat deposition in meal-feeding and nibbling rats. J. Nutr. 34:15-24, 1975.

57. VAUGHAN, D.A. and WINDERS, R.L. Effects of diet on HMP dehydrogenase and malic (TPN) dehydrogenase in the rat. Am. J. Physiol. 199:1041-1047, 1960.

58. MUIRURI, K.L., ROMSOS, D.R. and LEVEILLE, G.A. Influence of meal frequency on in vivo hepatic fatty acid synthesis, lipogenic enzyme activity and glucose tolerance in the chicken. J. Nutr. 105:963-971, 1975.

59. BERDANIER, C.D. Rat strain differences in response to meal feeding. Nutr. Reports Intl. 11:517-524, 1975.

60. BUSTAD, L.M. Swine in Biomedical Research. Seattle, Wash.: Frayn Printing Co., 1966.

61. MELNIKOV, S.V., and STRUK, I.I. O Kormlenija pri intensivom otkorme. Svinovodstvo. 8:25-29, 1957.

62. PSENCNYJ, P.D. Vliyanic kratkosti kormleniya na perevarimost i obmen vescestv u sviney. Vest. sel. khov. Nauki. 3:96-101, 1958.

63. PIDA (Pig Industry Development Authority). PIDA compares: pigs fed twice ad libitum and twice daily to appetite. A report on growth and caresses. PIDA, London, 1962.

64. CUPKA, V. and MAJERCIAK, P. Vplyu krmenia ad libitum na prirastky zivej vahy a jatocnu hodnotu vykrmovych osipanycn. Zivochisha vyroba 7:713-720, 1962.

65. FRIEND, D.W. and CUNNINGHAM, H.M. Effect of feeding frequency on metabolism, rate of gain and efficiency of gain on carcass quality of pigs. J. Nutr. 83:251-256, 1964.

66. FRIEND, D.W. and CUNNINGHAM, H.M. Growth, carcass, blood and fat studies with pigs fed once or five times daily. J. Anim. Sci. 26:316-322, 1967.

67. O'HEA, E.K. and LEVEILLE, G.A. Influence of feeding frequency on lipogenesis and enzymatic activity of adipose tissue and the performance of pigs. J. Anim. Sci. 28:336-341, 1969.

68. O'HEA, E.K. and LEVEILLE, G.A.: Lipogenesis in isolated adipose tissue of the domestic chick (Gallus Domesticus). Comp. Biochem. Physiol. 26:111-120, 1968.

69. ALLEE, G.L., ROMSOS, D.R., LEVEILLE, G.A. and BAKER, D.H. Metabolic adaptation induced by meal-eating in the pig. J. Nutr. 102:1115-1122, 1972.

70. ANDERSON, T.H. and FAUSCH, H.D. Effect of restricted access to feed on the serum and liver lipid levels of swine. Proc. Soc. exp. Biol. Med. 115:402-405, 1964.

71. COHN, C., PICK, R. and KATZ, L.J. Effect of meal-eating compared to nibbling upon atherosclerosis in chickens. Circulation Res. 9:139-145, 1961.

72. OKEY, R., SCHEIER, G., and REID, R. Food restrictions and cholesterol metabolism. J. Am. Dietet. A. 36:441-446, 1960.

73. WELLS, W.W., QUAN-MA, R., COOK, C.R., and ANDERSON, S.C. Lactose diets and cholesterol metabolism II. Effect of dietary cholesterol, succinyl-sulfathiazole and mode of feeding on atherosclerosis in the rabbit. J. Nutr. 76:41-47, 1962.

74. LEVEILLE, G.A. and HANSON, R.W. Influence of periodicity of eating in the chicken. Am. J. Physiol. 209:153-157, 1965.

75. LEPKOVSKY, S., CHARLI-BICRON, A., LEMMON, R.M., OSTWALD, R.C. and DIMICK, M.K. Metabolic and anatomic adaptations in chickens 'trained' to eat their daily food in 2 hours. Poult. Sci. 39:385-389, 1960.

76. FEIGENBAUM, A.S., FISCHER, H. and WEISS, H.S. Effect of 'meal-eating' vs 'nibbling' on body composition and digestive organ weight of normal and cropectomized chickens. Am. J. Clin. Nutr. 11:312-316, 1962.

77. O'HEA, E.K. and LEVEILLE, G.A. Lipid biosynthesis and transport in the domestic chick. Comp. Biochem. Physiol. 30:149-159, 1969.

78. COHN, C.: Feeding frequency and body composition. Ann. N.Y. Acad. Sci. 110:395-409, 1963.

79. VANPOTTEN, L.M., VANBEKKUM, D.W. and QUERIDO, A. Influence of hypothalmic lesions producing hyperphagia, and feeding regimens on carcass composition in the rat. Metab. 4:68-74, 1955.

80. MORIN-JOMAIN, M. and ABRAHAM, J. Comparison entire les effects de deux d'alimentation discontinue (sequences d'anition totale ou de jeune proteique) sur la croissance et la composition corporelle du rat blanc. Arch. Sci. Physiol. 19:131-139, 1965.

81. FÁBRY P., BRAUN, T., PETRÁSEK, R., HORÁKOVÁ, E. and KONOPASEK, E.: The effect of the pattern of food intake on the carcass composition of rats receiving diets with varying fat content. Physiol. Bohemoslov. 13:333-340, 1964.

82. FÁBRY P., PETRÁSEK, R., BRAUN, T., BEDNAREK, M., HORÁKOVÁ, E. and KONOPASEK, E. Lipogenesis in rats adapted to intermittent starvation or continuous underfeeding. Experientia 18:555-556, 1962.

83. WELCH, J.G. Effect of intermittent feeding on body composition of mice. Lab. Anim. Care 18:596-601, 1968.

84. MUIRURI, K.L. and LEVEILLE, G.A. Metabolic adaptation in meal-fed rats: effects of increased meal-frequency or ad libitum feeding in rats previously adapted to a single daily meal. J. Nutr. 100:450-460, 1970.

85. DEBONT, A.J., ROMSOS, D.R., TSAI, T.C., WATERMAN, R.A. and LEVEILLE, G.A. Influence of alterations in meal frequency on lipogenesis and body fat content in the rat. Proc. Soc. exp. Biol. Med. 149:849-854, 1975.

86. HEGGENESS, F.W. Effect of intermittent food restriction on growth, food utilization and body composition in the rat. J. Nutr. 86:265-270, 1965.

87. OZELCI, A., ROMSOS, D.R. and LEVEILLE, G.A. Influence of diet composition on nitrogen balance and body composition in meal-eating and nibbling rats. J. Nutr. 107:1768-1774, 1977.

88. ROMSOS, D.R. and LEVEILLE, G.A. Effect of intermittent feeding on body fat content and in vivo lipogenesis in mice. Nutr. Reports Intl. 5:131-138, 1972.

89. OZELCI, A., ROMSOS, D.R. and LEVEILLE, G.A. Influence of a liquid diet and meal pattern on body weight and body fat in rats. J. Nutr. 108:1128-1136, 1978.

90. OZELCI, A., ROMSOS, D.R. and LEVEILLE, G.A. Influence of initial food restriction on subsequent body weight gain and body fat accumulation in rats. J. Nutr. 108:1724-1732, 1978.

91. HOFFMAN, C.C., CARROLL, K.F. and GOLDRICK, R.B. Studies on lipid and carbohydrate metabolism in the rat. Aust. J. Exp. Biol. Med. Soc. 50:267-287, 1972.

92. COHN, C. and JOSEPH, D. Caloric intake, weight loss and changes in body composition of rats as influenced by feeding frequency. J. Nutr. 96:94-100, 1968.

93. CUTHBERTSON, D.P. Feeding patterns and nutrient utilization: Chairman's remarks. Proc. of the Nutr. Soc. 186th meeting, Edinburgh, Scotland, 1967, pp.143-190.

94. LEVEILLE, G.A. The long-term effects of meal-eating on lipogenesis, enzyme activity and longevity in the rat. J. Nutr. 102:549-556, 1972.

95. LEVEILLE, G.A. Glycogen metabolism in meal-fed rats and chicks and the time sequence of lipogenic and enzymatic adaptive changes. J. Nutr. 90:449-460, 1966.

96. ROBINSON, D.W., HODGSON, D., BRADFORD, G.E., RABB, J. and PETERSON, D.W. Effects of dietary restriction and fasting on the body composition of normally and genetically obese mice. J. Anim. Sci. 40:1058-1062, 1975.

97. MENDEZ, J. Effect of acute starvation and refeeding on body composition of rats fed previously at different levels of dietary protein. J. Nutr. 89:513-519, 1966.

98. COHN, C., SHRAGO, E. and JOSEPH, D. The effect of food administration on weight gains and body composition of normal adrenolectomized rats. Am. J. Physiol. 180:503-507, 1955.

99. WILSON, P.N. and FOSBOURNE, D. Compensatory growth after undernutrition in mammals and birds. Biol. Rev. 35:324-363, 1960.

100. STURDY, B.J., MARKS, V., KALUCY, R.S. and CRISP, A.H. Weight gain, thermic effect of glucose and resting metabolic rate during recovery from anorexia nervosa. Am. J. Clin. Nutr. 30:138-146, 1977.

101. SHARMA, V.D., YOUNG, L.G., BROWN, R.G., BUCHANAN-SMITH, J. and SMITH, G.C. Effects of frequency of feeding on metabolism and body composition of young pigs. Can. J. Anim. Sci. 53:157-164, 1973.

102. SCHOENBORNE, B.M. and CANOLTY, N.L. Influence of meal frequency on body composition and energy futilization in male weanling rats. Proc. Soc. Exp. Biol. Med. 163:10-14, 1980.

103. FAUST, I.M., JOHNSON, J.R., STERN, J.S. and HIRSCH, J. Diet-induced adipocyte number increase in adult rats: a new model of obesity. Am. J. Physiol. 3:235-286, 1978.

104. HIRSCH, J. and HANS, P.W. Cellularity of rat adipose tissue: effects of growth, starvation and obesity. J. Lipid Res. 10:77-82, 1969.

105. KNITTLE, J.L. and HIRSCH, J. Effect of early nutrition on the development of rat epididymal fat pads: cellularity and metabolism. J. Clin. Invest. 47:2091-2098, 1968.

106. BRAUN, T., KAZDOVÁ, L., FÁBRY, P., LOJDA, Z and HROMADKOVÁ, V. Meal-eating and refeeding after a single fast as a stimulus for increasing the number number of fat cells in abdominal adipose tissue of rats. Metab. 17:825-832, 1968.

107. ROMSOS, D.R. and LEVEILLE, G.A. Cellularity and in vitro Fatty Acid biosynthesis in adipose tissue of meal-fed and nibbling rat. Proc. Soc. Exp. Biol. Med. 139:868-871, 1972.

108. MOSINGER, B., KUJALOVÁ, V., and LOJDA, Z. Effect of the time distribution of food intake on the endocrine function of the pancreas. Cong. Fed. Inter. Diabetes 4th, Geneva, 1961.

109. COHN, C. and JOSEPH, D. Effects of caloric intake and feeding frequency on carbohydrate metabolism in the rat. J. Nutr. 100:78-84, 1970.

110. GWINUP, G., ROUSH, W., BYRON, R.C., KRUGER, K. and HAMUI, G.T. Effect of nibbling versus gorging on glucose tolerance. Lancet 2:165-169, 1963.

111. FÁBRY, P., HEJL, Z, FODOR, F., BRAUN, T. and ZVOLANKOVA, K.: The frequency of meals and its relationship to overweight, hypercholesterolemia and decreased glucose tolerance. Lancet 2:614-615, 1964.

112. NUNES, W.T., and CANHAM, J.E.: The effect of varied periodicity of eating on serum lipids and carbohydrate tolerance in man. Am.J. Clin. Nutr. 12:334, 1963.

113. BRAUN T., VRANA, A. and FÁBRY, P. Enhanced hypoglycemic effect of exogenous insulin associated with an increased response of adipose tissue and a diminished response of the diaphram in 'meal-fed' rats. Experentia 23:468-470, 1967.

114. SCHLIERF, G. and RAETZER, H. Diurnal patterns of blood sugar, plasma insulin, free fatty acids and triglyceride levels in normal subjects and in patients with type IV hyperlipoproteinemia and the effect of meal frequency. Nutr. and Metab. 14:113-126, 1972.

115. HOUSSAY, B.A. and MARTINEZ, C. Experimental diabetes and diet. Sci. 105:548-556, 1947.

116. COHN, C., PICK, R. and KATZ, L.N. Effect of meal-eating compared to nibbling upon artherosclerosis in chickens. Circulation Res. 9:139-145, 1961.

117. OKEY, R., SHANNON, A., TINOCO, J., OSTWALD, R. and MILJANICH, P. Fatty acid components of rat liver lipids: effect of composition of the diet and of restricted access to food. J. Nutr. 75:51-60, 1961.

118. ROMSOS, D.R., MILLER, E.R. and LEVEILLE, G.A. Influence of feeding frequency on body weight and glucose tolerance in the pig. Proc. Soc. Exp. Biol. Med. 157:528-530, 1978.

119. ROMSOS, D.R., BELO, P.J., BERGEN, W.G. and LEVEILLE, G.A. Influence of meal frequency on body weight, plasma metabolites and glucose and cholesterol metabolism in the dog. J. Nutr. 108:238-247, 1978.

120. GOPALAN, C., SPIKANTIA, S.G., JAGANNATHAN, S.N. and RAMANATHAN, K.S. Effect of the mode of feeding of fats on serum cholesterol levels and plasma fibrinolytic activity of monkeys. Am. J. Clin. Nutr. 10:332-336, 1962.

121. JAGANNATHAN, S.N., and GOPALAN, C. The influence of meal size on the effect of high fat diets on serum cholesterol in monkeys (Macaca radiata). Fed. Proc. 22:268, 1963.

122. OKEY, R., SCHEIER, G. and REID, R. Food restriction and cholesterol metabolism. J. Am. Dietet. A. 36:441-444, 1960.

123. FILLIOS, L., KAPLAN, R., MARTIN, R., SFTAVE, F. Gonadal regulation of cholesterol metabolism. Am. J. Physiol. 193:47-56, 1958.

124. FÁBRY, P., FODOR, J., HEJL, Z., GEIZEROVÁ, H., BALCAROVÁ, O. and ZVOLANKOVÁ, K. Meal frequency and ischaemic heart disease. Lancet 2:190-191, 1968.

125. IRWIN, M.I. and FEELEY, R.M. Frequency and size of meals and serum lipids, nitrogen and mineral retention, fat digestibility, and urinary thiamin and riboflavin in young women. Am. J. Clin. Nutr. 20:816-824, 1967.

126. COHN, C. Meal eating, nibbling and body metabolism. J. Am. Dietet. A. 38:433-436, 1961.

127. GWINUP, G., BYRON, R.C., ROUSH, W., KRUGER, F.A. and HAMUI, G.J. Effect of nibbling versus gorging on serum lipids in man. Am. J. Clin. Nutr. 13:209-213, 1963.

128. LEVEILLE, G.A. and O'HEA, E.K. Influence of periodicity of eating on energy metabolism in the rat. J. Nutr. 93:541-545, 1967.

129. HUENEMANN, R.L., and HAMPTON, M.C., SHAPIRO, L.R. and BEHNKE, A.R. Adolescent food practices associated with obesity. Fed. Proc. 25:4-10, 1966.

130. MAYER, J. Physical activity and anthropometric measurements in obese adolescents. Fed. Proc. 25:11-18, 1966.

131. JOHNSON, M.L., BURKE, B.S. and MAYER, J. Relative importance of inactivity and overeating in energy balance of obese high school girls. Am. J. Clin. Nutr. 4:37-44, 1956.

132. STEFANIK, P.A., HEALD, E.P. and MAYER, J. Caloric intake in relation to energy output of obese and nonobese adolescent boys. Am. J. Clin. Nutr. 7:55-62, 1959.

133. BULLEN, B.A., REED, R.B. and MAYER, J.: Physical activity of obese and non-obese girls appraised by motion picture sampling. Am. J. Clin. Nutr. 14:211-223, 1964.

134. GARNE, S.M. in personal communication, Michigan State University., May 1, 1980.

135. EPPRIGHT, E.S., FOX, H.M., FRYER, B.A., LAMKIN, G.H. and VIVIAN, V.M.: Northcentral regional study of diets and preschool children. 3. Frequency of feeding. J. Home Econ. 62:407-410, 1970.

136. LEON, G.R. A behavioral approach to obesity. Am. J. Clin. Nutr. 30:785-789, 1977.

137. COHN, C. and ALLWEISS, M.D. Fats, rats, chickens and men--results of feeding frequency. Am. J. Clin. Nutr. 12:255-265, 1963.

138. SWINDELLS, Y.E., HOLMES, S.H. and ROBINSON, M.F. The metabolic response of young women to changes in the frequency of meals. Brit. J. Nutr. 22:667-680, 1968.

139. HUENEMANN, R.L. Food habits of obese and non-obese adolescents. Postgrad. Med. 99:99-105, 1972.

140. DEUSTCH, R.M. The things kids eat. Family Guide to Better Food and Better Health. DesMoines, Iowa: Merideth Press, 1971, pp. 222-229.

141. DAMPWSLO. T.S., NOLAN, S. and STEPHAN, T. Obesity. World Review of Nutr. and Diet. 22:270-279, 1975.

NUTRITION RESEARCH, Vol. 1, pp. 551-552, 1981
0271-5317/81/050551-02$02.00/0 Printed in the USA.
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REVIEWS OF BOOKS

Annual Review of Nutrition

William J. Darby, Harry P. Broquist and Robert E. Olson, Editors. Vol. 1. Annual Reviews, Palo Alto, California. pp 509, 1981. Price $20.00 in U.S.A., $21.00 elsewhere.

Reviewers and readers learn about the launching of new series of collections of review articles with a sense of foreboding. Do we need another forum for assembling assays on different aspects of nutrition? Could the same material be obtained from review series in other areas of science and medicine? In what ways does the new series overlap or go beyond the 12 signed and about 90 unsigned reviews published in Nutrition Reviews? The contents and quality of contributions to Volume 1 of the Annual Review of Nutrition only partially allay such fears and questions. The book opens with a philosophical and erudite discussion of the moral dimension of world food supplies, an assay which ends on a pessimistic note. Earth metals and trace elements are covered in two chapters. Iron absorption and transport is discussed in relation to microorganisms and human data. Other chapters review flavoproteins, fructose and sugar alcohols, fluoride, regulation of energy balance, food colorants, cultural nutrition, conditioning of athletes, nutrition and toxicology, health effects of water. There is little evidence of any organized scheme for selection of topics and the reviews differ greatly in depth. The order in which the reviews are assembled in the volume is also highly irregular. There is a preponderance of articles on biochemical aspects of nutrition, reflecting perhaps the background and interests of the Editors of the volume. It is to be hoped that future volumes will achieve a better balance of subjects being tackled.

The new series of reviews in Nutrition is a welcome addition to the expanding literature in this field. The first volume gives us a glimpse of the vistas to expect in future editions. Departments of nutrition would be expected to subscribe to this series which is recommended; individuals would probably prefer to photocopy or order reprints of individual contributions if this is the best source of material in the subject that interests them.

RECENT ADVANCES IN OBESITY RESEARCH: III

Proceedings of the 3rd International Congress on Obesity. Per Bjorntorp, Michelangelo Cairella and Alan N. Howard, Editors. John Libbey, London. pp 392, 1981. L24.00.

The most prevalent nutritional problem in affluent industrialized societies is overnutrition and obesity. Every three years, researchers working in this area gather to exchange data. This volume contains the text of plenary talks given at the 3rd International Congress on Obesity held in Rome, 8-10 October, 1980. The section on hypertension in obesity discusses the mechanisms involved, particularly the handling of sodium and endocrinal changes, and outlines the principles of management. Four papers address the question of adipocyte development and turnover and how this might influence the management of obese individuals. There are articles on the prognosis of obese subjects

undergoing weight reduction programs and whether the relapsing patient can be identified. The relationship of tissue lipoprotein lipase and obesity is discussed as is refeeding effects on adipocyte metabolism. Several papers review the techniques and pitfalls of measurement of energy expenditure. Another section summarizes the drug treatment of obese individuals, including the role of adjunct behaviour modification. Concepts of thermogenesis and thyroid hormones are espoused and data related to insulin resistance are reviewed. Two sections analyze the effect of physical training and exercise on weight control and allude to the complications which may occur occasionally.

The quality of a volume which records the proceedings of a conference must necessarily depend upon the academic merit of various individual papers. This volume achieves a surprising continuity in discussions, which is helped to some extent by high quality reviews interspersed throughout the book. These include Indications for the treatment of obesity by M. Berger and colleagues, Psychological factors in obesity by Judith Rodin, Experimental obesities by Bernard Jeanrenaud and coworkers, The phenomenon of satiety by Theodore van Itallie and Dennis VanderWeele, Surgical treatment of obesity by Flemming Quaade and Is corpulence catching? by George Bray. The occasional editorial lapse, such as the inclusion of an abstract on p.172, does not detract from the general good quality of the material included in this volume. It is to be hoped that additional aspects of obesity not covered in previous congresses, such as Fetal aspects, Immunological mechanisms, and the special problems of obesity among children and adolescents, would merit inclusion in future conferences.

NUTRITION RESEARCH, Vol. 1, pp. 553-554, 1981
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SUMMARIES OF CONFERENCES

XII International Congress of Nutrition

Topics discussed at the plenary symposia held during the Congress were reviewed in the last number of Nutrition Research. Data presented during free communication and poster sessions are selectively summarized below.

TORUN studied the nutritional adequacy of traditional Central American foods such as black beans, corn, bread and vegetables fed ad libitum plus small fixed amounts of egg, milk or meat fed some days of the week. Mean daily protein and energy intakes were 1.92 g and 91 kcal respectively per kg body weight. Overall nitrogen balance was positive in all children and growth was adequate. It was concluded that predominantly vegetable-based diets can fulfill the nutritional requirements of preschool children provided there the amounts are sufficient to satisfy appetites, energy density of some foods is increased, and there is adult supervision to encourage children to eat. FUENTES carried out a retrospective study of hospitalized children and correlated recovery with frequency of febrile illness. It was found that nutritional supplements resulting in correction of malnutrition also resulted in fewer episodes of fever. FIOROTTO studied the effects of protein restriction on sodium and water retention in infant baboons and found a reduction in renal tubular capacity. There was areduction in blood volume and and a rise in plasma renin activity and aldosterone concentration. Reflex mechanisms could obviously minimize sodium and water wastage by regulating the amount of fluid available to kidneys for filtration but at plasma oncotic pressures below a threshold value, fluid filtration into the interstitium took precedence over its filtration in the kidney facilitating the formation of edema. OFTEDAL measured energy intake among the suckling young of horse, sheep, dog, striped skunk, mink and guinea pig, and a linear correlation was obtained with body weight, r=0.996). ABDULLA studied 60 women from the above 95th and below 30th percentiles and estimated energy and salt intakes. The mean values for most nutrients and salt did not differ in the two groups with high and low blood pressure.

ALTCHULER measured changes in urinary calcium and hydroxyproline in children with Crohn's disease before and after giving a high-protein diet. There was a significant increase in hydroxyproline excretion after nutritional supplementation and this correlated with catch-up growth. OLOWOOKERE investigated energy transduction features of mitochondrial coupling membrane protein-energy malnutrition and found evidence for less coupling in the deprived group. HEYWOOD conducted a prospective study of children in the Tari basin of Papua New Guinea and found that the prospective. risk of death increased as weight-for-age, height-for-age and

weight-for-height deacreased. RAHAMAN studied hospitalized children in Bangladesh and observed progressive increase in mortality rate from 3.7% in those with no evidence of malnutrition 5.4% in those with mild malnutrition and 14.3% in the ones with severe nutritional deficiency.

ALLEN used a specially designed questionnaire to survey the incidence and duration of breast feeding in three developing countries. In Kenya, 80% of infants were being breast-fed at 12 months of age. However, indigenous cereals are often fed to the babies in early infancy. In Malaysia, only 40% of the mothers were breast feeding their infants at 12 months. There are marked ethnic differences, only half of the Chinese mothers breast feed their infants. The most common reason for supplementation and giving solids was inadequacy of breast milk. REA investigated the effect of social and work conditions on breast feeding patterns in urban Sao Paulo. Breast feeding was initiated in at least 86% of all mothers but the mean duration of exclusive breast feeding was short, 3.5-4.7 weeks. This was correlated with family instability, socioeconomic insecurity and adverse labor conditions. VILLAVIEJA studied feeding and weaning practices in Filipino infants and observed that colostrum was given to 60% of infants, a practice unaffected by mother's age and education. 86% were breast fed at 12 months. Solids are introduced at a mean age of 5.8 months and the infants are weaned at 10 months. Interestingly, weight-for-age was better in the breast fed group till 6 months of age but not beyond that age period. SAMUELS found that only 39% of women were breast feeding at 4 months of age in an American community. Solimano compared morbidity patterns and found that breast fed infants experienced lower morbidity even in an urban U.S. setting. ACOSTA calculated the human lactation cost which varied from $2.67 to $1.14 per day depending upon the energy derived from biokost.

A number of papers looked at the nutrition of the elderly. STEEN used a whole body counter to measure total body potassium and tritiated water to measure total body water, at ages 70, 75 and 79 years. In both sexes, height and weight as well as the absolute amounts of body cell mass and extracellular water decreased and that of body fat increased. RUDAS suggested that changes in taste acuity may be responsible in part for altered dietary intake in the elderly. ZAMENOHOF found considerably altered mortality pattern and life span depending upon when chronic undernutrition was induced. EVERITT presented data implicating the anterior pituitary in food-related effects on the ageing process. IRITANI the turnover and Km of lipogenic enzymes and found age-dependent alterations in the former.

Among other ares of nutrition covered by free communications and poster sessions were drug-nutrient interactions, ruminant nutrition, obesity, poultry and animal nutrition, trace elements including zinc, nutrition and cancer, reproduction and cardio-vascular disease, macronutrients, vitamins, nonnutrient dietary constituents, assessment of nutritional status, and nutrition in national policy and planning.

NUTRITION RESEARCH, Vol. 1, pp. 555, 1981
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ANNOUNCEMENTS

International Workshop on Nutrition and the Development of the Child

This meeting sponsored by a number of international agencies and the University Grants Commission of India, is scheduled to be held in Baroda, India, January 9-15, 1982. Plenary talks, workshops and free communications are planned. For further information, contact Dr. C.V. Ramakrishnan, Department of Biochemistry, M.S. University of Baroda, Baroda 390002, India.

International Food Allergy Symposium IV

The American College of Allergists is sponsoring the symposium which will be held July 24-29, 1982, in Vancouver, British Columbia, Canada. Plenary lectures, luncheon seminars and free papers are planned. The symposium chairman is James C. Breneman and further information may be obtained from him at 9880 East Michigan Avenue, Galesburg, MI 49053 or from the Executive Secretary of the College, Shirley Schoenberger, 2142 14th Street, Boulder, CO 80302.

CHEMRAWN II

The International Conference on Chemistry and World Food Supplies - The New Frontiers will be held February 2-6, 1982 in Manila, Philippines. For further information contact CHEMRAWN II, International Food Policy Research Institute, 1776 Massachusetts Avenue, Washington DC 20036.

International Symposium on Nutritional Problems in Childhood

The University of Modena is sponsoring a meeting in which selected aspects of childhood nutrition will be discussed. The program includes vitamins, trace elements, lipids, intolerance and allergy, diabetes, obesity and endocrines. For further information, contact Professor O. Olivi, First Clinica Pediatrica, Dell'Universita di Modena, Modena, Italy.

Nutritional Support of Specific Disorders

This symposium sponsored by the City of Hope National Medical Center will be held at the Huntongton-Sheraton Hotel in Pasadena, California, March 6-7, 1982. The program includes Assessment of nutritional status, Nutritional management in gastrointestinal disease, Nutrition of the patient with renal disease, Psychological impact of food on the life cycle, Nutritional management of the cancer patient, Does nutrition affect nutritional status?, Effect of nutrition on brain function, Nutritional support of the trauma patient, and Anorexia nervosa. Specific cases will be discussed in panel discussions and workshops are planned. For further information and registration, contact Dr. Michael M. Meguid, City of Hope National Medical Center, 1500 East Duarte Road, Duarte, CA 91010.

NUTRITION RESEARCH, Vol. 1, pp. 557-558, 1981
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EDITORIAL

Nutrition in Old Age

The inevitability of aging and death has intrigued both scientists and philosophers. The transition to old age is associated with a progressive loss of resistance to disease. However, the biologic effects of aging are observed at widely different chronologic ages. Lifetime experiences, including dietary intake, have cumulative impact on body composition and function. Aging changes taste acuity, appetite, physiology and metabolism (1,2). Nutritional requirements in the elderly must of necessity be different. Thus it is important to document the nutritional status of old people. Some recent studies have addressed this question and have found nutritional deficits in a significant proportion of people above the age of 60 years (3-5). Many of the diseases of the elderly, including infection, cancer, autoimmunity, and amyloidosis, point to declining immunocompetence. At the same time, there is atrophy of tissues, and anthropometric and biochemical evidence of malnutrition. We know that nutritional deficiency results in profound changes in lymphoid organ morphology and immune responses (6). Thus, it is possible that nutrition changes contribute to increased morbidity in old age. This is potentially salvagable.

Improvements in socioeconomic and health conditions in the last four decades have ensured that many people in affluent societies will escape the biologic and health hazards of childhood and adult life to reach old age. In North America alone, at least 24 million individuals are above the age of 60 years. It is imperative that health professionals and scientists identify the nutritional needs and problems of this important segment of the population so that appropriate dietary measures can be adopted to prevent and reduce nutrition-related morbidity and mortality in the elderly.

R.K. Chandra

1. MASORO, E.J. Physiologic changes with aging. In: Nutrition and Aging. M. Winick (ed). Wiley, New York, 1976, pp. 61-76.

2. NEIGE TODHUNTER, E. Nutrition of the elderly. In: Human Nutrition. 3b. Nutrition and the Adult. R.B. Alfin-Slater and D. Kritchevsky (eds). Plenum, New York, 1980, pp. 397-417.

3. LEE, C.J., JOHNSON, G.H. and LAWLER, G.S. Some clinical indices of nutrition and health status among elderly Kentucky residents volunteering to be surveyed. Nutr. Res. 1: 47-62, 1981.

4. CHANDRA, R.K. Nutrition and immunocompetence of the elderly. J. Amer. Coll. Nutr. Volume 1, January 1982 (in press).

5. WAGNER, P.A., BAILEY, B., KRISTA, M.L., JERNIGAN, J.A. and CERDA, J.J. Comparison of zinc and folacin status in elderly women from different socioeconomic backgrounds. Nutr. Res. 1:565-570, 1981.

6. CHANDRA, R.K. Immunodeficiency in undernutrition and overnutrition. Nutr. Rev. 39: 225-231, 1981.

NUTRITION RESEARCH, Vol. 1, pp. 559-564, 1981
0271-5317/81/060559-06$02.00/0 Printed in the USA.
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A LOWERING EFFECT OF A PHARMACOLOGICAL DOSE OF VITAMIN E ON SERUM VITAMIN A IN NORMAL ADULTS

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University of Maryland School of Medicine and the Veterans Administration Medical Center

ABSTRACT

We have studied the effects of oral vitamin E (vE) on serum vitamin A (vA) levels and vA transport proteins, retinol binding protein (RBP) and prealbumin (PA), in normal subjects taking and not taking pharmacological doses of vA and/or vE acetate. Nineteen subjects were given 9009 ug of oral vA acetate daily for one week. Ten of these continued on 9009 ug of vA daily for two weeks (group I), while nine received 147 mg of vE acetate in addition to vA (group II). A third group (group III) consisted of 10 subjects who received only 147 mg of vE daily for 3 weeks. Group IV represented control subjects not given either vA or vE pills over the 3 week study period. Groups I and II showed significant increases in serum vA levels after 1 week of vA treatment. Group I then continued to show a significant rise in serum vA levels from week 1 to week 3 ($p<0.01$) while group II serum vA levels significantly fell from week 1 to week 3 ($p<0.01$). No significant differences were demonstrated in RBP or PA in groups I and II. Group III showed a significant fall in serum vA and RBP levels from initial to week 3 ($p<0.005$) with no significant change in PA. Control subjects (group IV) showed no significant variation of any parameter over the three week test period. The fall in serum vA and RBP seen among our subjects taking vE alone is consistent with either enhanced hepatic vA uptake or increased vA transport to and utilization by peripheral tissues.

Key words: Vitamin E, vitamin A, vitamin A absorption, vitamin A metabolism.

INTRODUCTION

A relationship between the metabolism of vitamin A (vA) and vitamin E (vE) was first described in the early 1940's (1-4). Because of the antioxidant properties of vE and because performed and precursor vA active compounds are easily oxidized, a sparing effect of vE (tocopherols) on vA has been a generally accepted theory of interaction. This effect is thought to occur both in the intestinal lumen during digestive and absorptive processes (5,6) and at the cellular level in tissues remote from the gastrointestinal tract (1).

More recent information suggests other roles for vE, e.g. the promotion of vA uptake and utilization by body tissues (5). We have studied the effect of oral vE and serum vA and vA transport protein levels (retinol binding protein (RBP) and prealbumin (PA)) in normal subjects taking and not taking vA and/or vE in pharmacological doses.

METHODS

Thirty-seven normal adult subjects (16 female and 21 male) ranging in age from 20 to 35 years (mean age ± SD = 28 ± 2) were studied. No subject was taking any medication and none gave a history of recent illness. Baseline fasting blood samples were obtained from all subjects for the following serum determinations: vA, vE, RBP, and PA.

Serum vA analysis was determined by the trifluoroacetic acid method of Neeld and Pearson (7) (our laboratory normal range based on the mean ± SD values of 120 normal individuals = 27 - 83 ug/dl). Serum vE was measured using the colorimetric reaction of Martinek and Quaife (normal range = 5 - 15 ug/ml)(8,9). Serum RBP and PA levels were analyzed using M-partigen radioimmunodiffusion plates (Calbiochem-Behring Diagnostics, Hoechst, Somerville, NJ). The normal RBP and PA ranges for our laboratory are 29-81 ug/ml and 16-41 mg/dl, respectively.

Following baseline testing, nineteen subjects were given 9009 ug (30,000 IU) or oral vA acetate daily for one week. Ten of these subjects then continued on 9009 ug of vA daily for two weeks (group I) while nine received 147 mg (200 IU) or oral vE acetate in addition to vA (group II). A third group (group III) consisted of ten subjects who received only 147 mg of vE daily for three weeks. For comparison, eight control subjects were not given either vA or vE pills during the three week study period (group IV).

Statistical calculations were based on both paired and unpaired Student's t-test. The level of significance is taken at a value of $p=0.05$.

RESULTS

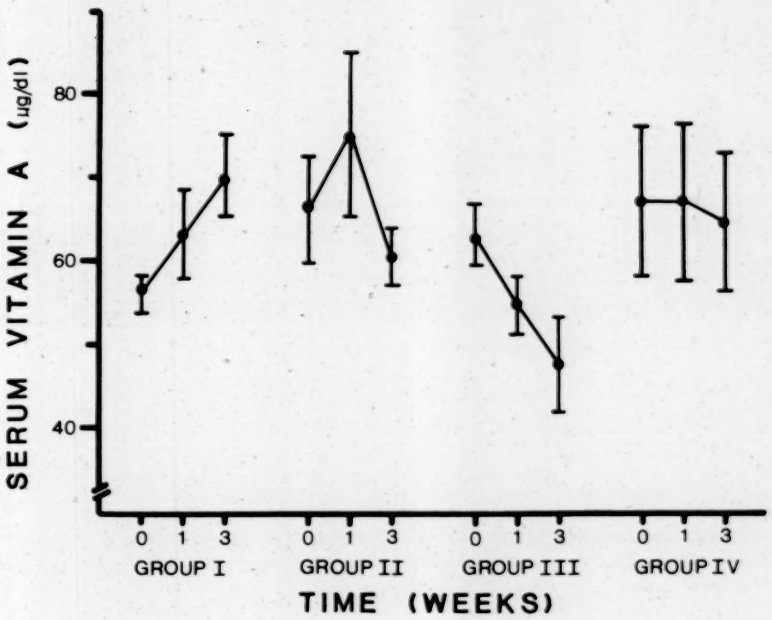
There was no significant difference in initial values for any parameter in any treatment group. Groups I and II showed significant increases from baseline in serum vA levels after one week of vA treatment ($p<0.05$)(Fig. 1). Group I (given vA alone) then continued to demonstrate a rise in serum vA from week 1 (mean ± SEM = 63 ± 3 ug/dl) to week 3 (70 + 5 ug/dl, $p<0.01$) whereas group II (given vE at 1 week) showed a fall in serum vA level from week 1 (mean ± SEM = 75 ± 10 ug/dl) to week 3 (60 ± 4 ug/dl, $p<0.01$). Serum vE did not change in group I from week 1 to week 3, whereas it significantly rose in group II from week 1 to week 3 ($p<0.001$)(Table 1). There was no difference in serum vA or vE levels between groups I and II at week 1 or week 3. Also, Groups I and II showed no significant changes in RBP or PA levels throughout the study period.

Group III (given vE alone) demonstrated a continual and significant decline in serum vA levels from initial testing (mean ± SEM = 63 ± 4 ug/dl) to 3 weeks (54 ± 4 ug/dl, $p<0.005$)(Fig. 1). In this group, serum vE levels rose from initial to week 1 ($p<0.001$) but did not significantly change thereafter. Concurrently, serum RBP levels fell from week 1 to week 3 ($p<0.005$). There was no change in serum PA levels in group III.

Control subjects (given neither vA nor vE) showed no significant variation in any parameter over the three week test period.

Fig. 1

Serum Vitamin A Levels (Mean ± SEM) at 0, 1, and 3 Week Intervals for Three Experimental Groups and One Control Group



Group I (n=10), given 9009 ug oral vA daily for 3 weeks; Group II (n=9), given 9009 ug oral vA daily for 1 week at which time 147 mg oral vE was added daily for 2 weeks; Group III (n=10), given 147 mg oral vE faily for 3 weeks; Group IV (n=8), represents control subjects not given either vA or vE pills during the 3 week test period.

DISCUSSION

It has been shown in experimental animals that vE enhances the intestinal absorption (4,6) and liver storage (3,11) of vA and, further, that vE may be essential for normal in vivo utilization of vA(6). Among subjects given vE alone (group III), we observed a significant fall in serum vA and RBP levels concurrent with a rise in serum vE levels. Our results are consistent with either enhanced hepatic uptake or increased vA transport and utilization by target tissues. That is, the

TABLE 1

Concentrations (Mean + SEM) or Serum Vitamin E (vE), Retinol Binding Protein (RBP) and Prealbumin (PA) at Initial, One, and Three Week Intervals in Three Experimental groups and a Control Group *

Group	Time Intervals	Serum Levels vE (ug/ml)	RBP (ug/ml)	PA (mg/dl)
I	Initial	9.4 ± 0.4	56 ± 2	29 ± 1
	1 Week	9.9 ± 1.2	57 ± 4	30 ± 2
	3 Weeks	10.3 ± 0.5	55 ± 7	30 ± 2
II	Initial	8.9 ± 0.9	61 ± 5	32 ± 3
	1 Week	9.5 ± 0.6	58 ± 5	32 ± 3
	3 Weeks	12.2 ± 1.1 **++	60 ± 5	31 ± 2
III	Initial	9.0 ± 0.7	53 ± 3	27 ± 2
	1 Week	12.4 ± 1.20 ***	53 ± 3	28 ± 2
	3 Weeks	12.3 ± 13.0 ***	48 ± 3**++	26 ± 1
IV	Initial	7.6 ± 1.0	59 ± 9	31 ± 3
	1 Week	8.0 ± 1.0	58 ± 2	28 ± 3
	3 Weeks	8.6 ± 1.5	56 ± 9	30 ± 2

*Group I received 9009 ug oral vA daily for 3 weeks; Group II received 9009 ug oral vA daily for 1 week at which time 147 mg oral vE was added daily for 2 weeks; Group III received 147 mg oral vE daily for 3 weeks; Group IV was not given vA or vE during the 3 week test period.

**p<0.005; ***p<0.001 compared to initial values
++p<0.005; +++p<0.001 compared to 1 week values

liver could be sequestering vA without concomitant secretion of vA and RBP into the bloodstream. Alternatively, vE may cause increased delivery of vA to peripheral tissues with subsequent utilization. After delivery of vA, apo-RBP, with its reduced affinity for PA, may be excreted by renal glomeruler filtration at an increased rate (12).

Increased peripheral utilization of vA may be due to a sparing effect of vE at the molecular level by reducing oxidative destruction of vA. An increased availability of vA to peripheral tissues (when given with vE) may result in more rapid improvement of signs and symptoms of clinical vA deficiency (15). Over prolonged periods, however, improved availability to target tissues could result in faster depletion of hepatic vA stores unless vE has a beneficial effect at this storage site as well.

Studies in children by Kusin et al (10) have shown that administration of large doses of vE had no effect on absorption or retention of vA. Moreover, Jagadeesan et al (16) showed no correlation between plasma vA and vE levels in children. Conversely, Ekvall et al (17) demonstrated a significant increase in post-prandial levels of serum vA and vE following simultaneous ingestion of vA and vE in children with cystic fibrosis of the pancreas. The conflicting results between animals and human studies may be due to differences in the quantity of vE administered, the chemical form of each vitamin, the method of administration, the site(s) of metabolic interaction and/or other dietary factors (e.g. the intake of dietary fat)(11). Moreover, animal models may not be representative of human vA-vE interaction. Further studies are needed to specifically examine the mechanisms and possible sites of vA and vE interaction using radiolabelled vA compounds in appropriate models.

In this study short term (1-3 weeks) vA treatment at a moderately high vA dose (9009 ug/day) resulted in a significant rise in serum vA, although vA values remained within normal limits. Without a concurrent rise in serum RBP, the increase in circulatory vA may represent an increase in retinyl esters, which were not measured (18). It is possible that a vA sufficient liver requires time to adapt its efficiency of vA uptake when presented with a daily vA load which is moderately elevated (~6 fold). However, it is unlikely that serum vA values would rise further on continued vA treatment; rather a return to baseline vA values might be expected.

REFERENCES

1. GREEN, J., MUTHY, I.R., DIPLOCK, A.T., BUNYAN, J., CAWTHORNE, M.A. and MURRELL, E.A. Vitamin E and stress. 7. The interrelationships between polyunsaturated fatty acid stress, vitamin A and vitamin E in the rat and chick. Br. J. Nutr. 21: 845-864, 1967.

2. HARRILL, I., MINARIK, G., and GIFFORD, E.D. Effect of vitamin A and E on lipids in selected rat tissues. J. Nutr. 87: 424-428, 1965.

3. MOORE, T. The effect of vitamin E deficiency on vitamin A reserves of the rat. Biochem J. 34: 1321-1328, 1940.

4. HICKMAN, K.C.D., KALEY, M.W., and HARRIS, P.L. Covitamin studies. III. The sparing equivalence of the tocopherols and mode of action. J. Biol. Chem. 152: 321-328, 1944.

5. TAPPEL, A.L. Vitamin E and free radical peroxidation of lipids. N.Y. Acad. Sci. Ann. 203: 14-28, 1972.

6. AMES, S.R. Factors affecting absorption, transport, and storage of vitmain A. Am. J. Clin. Nutr. 22: 934-935, 1969.

7. NEELD, J.B., and PEARSON, W.N. Macro- and micromethods for the determination of serum vitamin A using trifluoroacetic acid. J. Nutr. 79: 454-462, 1963.

8. MARTINEK, R.G. Method for the determination of vitamin E (total tocopherols) in serum. Clin. Chem. 10: 1078-1086.

9. QUAIFE, M.L., SCRIMSHAW, N.S., and LOWRY, O.H. A micromethod for assay of total tocopherols in blood serum. J. Biol. Chem. 180: 1229-1235, 1949.

10. KUSIN, J.A., REDDY, V., and SIVAKUMAR, B. Vitamin E supplements and the absorption of a massive dose of vitamin A. Am. J. Clin. Nutr. 27: 774-776, 1974.

11. SONDERGAARD, E. The influence of vitamin E on the expenditure of vitamin A from the liver. Experimentia 28: 773-774, 1972.

12. SMITH, J.E., and GOODMAN, D.S. Retinol-binding protein and the regulation of vitamin A transport. Fed. Proc. 38: 2504-2509, 1979.

13. ROBINSON, W.G., JR., KUWABARA, T., and BIERI, J.G. Deficiencies of vitamins E and A in the rat. Invest. Ophthamol. Vis. Sci. 19: 1030-1037, 1980.

14. ROBINSON, W.G., JR., KUWABARA, T., and BIERI, J.G. Vitamin E deficiency and the retina: photoreceptor and pigment epithelial changes. Invest. Ophthalmol. Vis. Sci. 18: 683-690, 1979.

15. McLAREN, S.D., SHIRAJIAN, E., TCHALIAN, M., and KHOURY, G. Xerophthalmia in Jordon. Am. J. Clin. Nutr. 17: 117-130, 1965.

16. JAGADEESAN, V., and REDDY, V. Interrelationship between vitamins E and A: A clinical study. Clin. Chem. Acta. 90: 71-74, 1978.

17. EKVALL, S, and MITCHELL, A. The effect of supplemental vitamin E on vitamin A serum levels in cystic fibrosis. Internat. J. Vit. Nutr. 48: 325-332, 1978.

18. SMITH, F.R., and GOODMAN, D.S. Vitamin A transport in human vitamin A toxicity. N. Eng. J. Medicine 15: 805-808, 1976.

ACKNOWLEDGEMENTS

We are indebted to Ms. Helen Spencer and Mr. David Twomey for valuable assistance in the preparation of the manuscript, and to Ms. Valerie Lowe and Dr. Robert Jacob for essential technical assistance.

This research was supported by the NIH Grant No. 7 R22 AM31081-01 (U.S.-Japan Cooperative Program).

NUTRITION RESEARCH, Vol. 1, pp. 565-569, 1981
0271-5317/81/060565-05$02.00/0 Printed in the USA.
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COMPARISON OF ZINC AND FOLACIN STATUS IN ELDERLY WOMEN FROM DIFFERING SOCIOECONOMIC BACKGROUNDS[1-3]

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ABSTRACT

Zinc and folacin status of 132 women, aged 55-87, from differing socioeconomic backgrounds was evaluated based on concentrations of zinc in hair and of folacin in erythrocytes. Elderly women of middle and upper socioeconomic status (Group I) had higher ($p < 0.001$) hair zinc and erythrocyte folacin levels than those observed in women of lower socioeconomic status (Group II). The prevalence of low values for Group I and II respectively was: 3%, 29% (hair zinc< 100 ug/g); and 6%, 60% (erythrocyte folacin< 140 ng/ml). These findings demonstrate that the risk of zinc and folacin inadequacies is greater among elderly women of lower socioeconomic status.

KEY WORDS: Zinc, folacin, elderly women, nutritional status

INTRODUCTION

Recent attention to the social and health problems of older Americans has led to concern that certain segments of the elderly population may be at increased risk of malnutrition and its related health consequences (1,2). Evidence is accumulating that zinc and folacin deficiencies among the elderly may be more common than previously suspected (3,4). Work from our laboratory suggests that zinc and folacin status of elderly persons from urban low-income households may be less than adequate (5,6). However, interaction of nutrition with the aging process is complex and lower laboratory values in the elderly may not necessarily represent nutritional deficiencies (2,7).

The possibility that inadequate zinc nutrition may be one factor contributing to poor folacin status in the older adult should also be recognized. Impairment of polyglutamyl folate utilization in zinc deficient young men (8) and the observation that bovine hepatic folate conjugase is a zinc metalloprotein (9) suggest that zinc

[1]Supported in part by NIH - National Institute on Aging Grant R01 AG00833-02.
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is required for the absorption of dietary polyglutamyl folates. Severe impairment of dietary polyglutamyl folate utilization has been observed in elderly individuals (10). In contrast, absorption of monoglutamyl folates by these elderly subjects was similar to that of younger adults.

While data from national surveys suggest that persons of lower socioeconomic status are at increased risk of malnutrition (11,12,13), this factor has not been examined with regard to laboratory indices of zinc and folacin status in the elderly population. The present report compares hair zinc and erythrocyte folacin levels in elderly women from differing socioeconomic backgrounds. These indices were selected because they are considered more representative of long-term nutritional status than plasma or serum levels (3,14,15). Evidence is presented that the risk of zinc and folacin inadequacies is greater among those of lower socioeconomic status.

METHODS

Subjects. The sample included 132 non-institutionalized, free-living women, aged 55-87 from two separate communities. Seventy-five percent were between the ages of 60-75. Group I consisted of 41 white women from middle and upper socioeconomic backgrounds who resided in a retirement community noted for its emphasis on preventive health care. Women accepted as study participants from this community were judged to be free of significant clinical disease as evaluated by medical histories and standard laboratory tests including complete blood count, urinalysis, stool guaiac, SMAC 23, electrocardiogram and an intermediate strength PPD test. None of these women were taking folacin supplements and only three reported use of a daily zinc supplement of 10-30 mg. Group II included 93 black women from a densely populated urban poverty area. Study participants were volunteers recruited by community workers and more than 90% of the subjects were from households with incomes near or below the poverty income threshold level. Use of food stamps was reported by 51% of the households surveyed and the average food shopping frequency was less than two times per month. Subjects were examined by a physician skilled in the diagnosis of nutritional disorders. Although 20% of the subjects reported use of some type of vitamin or mineral supplement, these did not include zinc or folacin. Protocol for this study was approved by the University of Florida Committee for the Protection of Human Subjects and informed consent was obtained from all subjects.

Sample collection and analysis. Samples of hair and blood were collected and analyzed for zinc and folacin, respectively, according to our previously described protocol (5,6). Hair was cut close to the scalp from the suboccipital area of the head and only the proximal 2 cm portion was retained in order to obtain more recently grown hair for zinc analysis. Hair samples were washed, wet ashed and analyzed for zinc by atomic absorption spectrophotometry as outlined by Sorenson et al. (16). Subjects with hair zinc levels below 100 ug/g were considered to be at increased risk of zinc inadequacy (14). Blood samples were collected by venipuncture in the morning after an overnight fast, placed in tubes containing ascorbic acid, protected from light and analyzed for folacin microbiologically with *Lactobacillus casei* using the methods described by Herbert et al (17) and Hoffbrand et al. (18). Subjects with erythrocyte folacin levels below 140 ng/ml were considered to be at risk of folacin deficiency (15). The computer based Statistical Analysis System (SAS) and the facilities of the Northeast Regional Data Center of the State University System of Florida located on the campus of the University of Florida in Gainesville were utilized for statistical analysis of the data (19).

RESULTS

Zinc and folacin status of 132 women, aged 55-87, from differing socioeconomic backgrounds was evaluated based on hair zinc and erythrocyte folacin levels presented in Table 1. Elderly women of middle and upper socioeconomic status (Group I) had higher ($p < 0.001$) hair zinc and erythrocyte folacin levels than those observed in women of lower socioeconomic status (Group II). The prevalence of low values for

both micronutrients in Group II was approximately tenfold greater than in Group I. In Group II, 29% had hair zinc levels below 100 ug/g, in contrast to only 3% in Group I and erythrocyte folacin levels were below 140 ng/ml in 60% of Group II women as compared to only 6% in Group I.

TABLE 1

Hair Zinc and Erythrocyte Folacin Levels in Women, Aged 55-87, from Differing Socioeconomic Backgrounds

Group	I	II
Socioeconomic Status	Middle & Upper	Lower
Hair Zn		
No. of subjects	39	93
Mean ±S.D. (ug/g)	187±47[a]	144±80[b]
Prevalence of levels <100 ug/g (%)	3[a]	29[b]
Erythrocyte folacin		
No. of subjects	41	83
Mean ± S.D. (ng/ml)	500±362[a]	168±93[b]
Prevalence of levels <140 ng/ml (%)	6[a]	60[b]

[a,b]Values not sharing a common superscript are significantly different ($p < 0.001$)

DISCUSSION

Our comparison of laboratory indices of zinc and folacin status in elderly women from differing socioeconomic backgrounds demonstrates that the risk of these micronutrient inadequacies is greater among women of lower socioeconomic status. This conclusion is in agreement with data from national surveys which suggest that persons of lower socioeconomic status are at increased risk of malnutrition (11,12,13).

It should be recognized that in addition to the socioeconomic variable, our study involves a comparison of black and white women. Any interpretation of differences in biochemical indices between racial groups should take into account possible genetic as well as nutritional factors. For example, hemoglobin norms are thought to be slightly lower for black population groups (20). While genetic factors may contribute, we propose that the marked difference in zinc and folacin levels between the two groups of women in our study are more likely due to the known influence of socioeconomic status on nutritional risk. This conclusion is supported by the fact that no effect of race on hair zinc levels could be demonstrated in a survey of black and white elderly individuals from similar socioeconomic backgrounds (3).

An examination of food frequency data suggests that women in the lower socioeconomic group had very low intakes of foods considered to be good sources of folacin or zinc. For example, fresh vegetables were included on a daily basis by only 17% of the group and animal protein sources were consumed less frequently than legumes. In contrast, all major food groups were represented

in the daily diets of women from middle and upper socioeconomic backgrounds, who were apparently able to maintain hematological indices, erythrocyte folacin and hair zinc levels comparable to those found in younger adults, without the use of supplemental folacin or zinc. These findings lend support to the concept that available laboratory norms developed for younger adults are generally acceptable for evaluating nutritional status in the elderly population.

No correlation between zinc and folacin levels could be demonstrated in either group of elderly women. However, further work is needed to clarify the practical importance of the postulated role of zinc in absorption of dietary polyglutamyl folates (8).

In summary, the high prevalence of reduced erythrocyte folacin and hair zinc levels in elderly women of lower socioeconomic status suggests that tissue stores of these micronutrients are being depleted. While much remains to be learned about the functional consequences of this depletion in the older adult, it seems desirable to identify those at risk of malnutrition prior to the onset of clinically evident disease. Our comparison of laboratory findings in elderly women from differing socioeconomic backgrounds demonstrates that the risk of zinc and folacin inadequacies is greater among those of lower socio-economic status.

REFERENCES

1. BRUN, J. K. and CLANCY, K. L. Low-income and elderly populations. J. Nutr. Ed., 12 (suppl. 1, no. 2), pp. 128-130, 1980.

2. WATKIN, D. M. Better Nutrition for those already old: the challenge of the eighties. Aging, 311-312: 21-28, 1980.

3. GREGER, J. L. and SCISCOE, B. S. Zinc nutriture of elderly participants in an urban feeding program. J. of Am. Dietetic Assoc., 70: 37-41, 1977.

4. BAKER, H., JASLOW, S. P. and FRANK, O. Severe impairment of dietary folate utilization in the elderly. J. of Am. Geriatrics Soc., 26: 218-221, 1978.

5. WAGNER, P. A., KRISTA, M. L., BAILEY, L. B., CHRISTAKIS, G. J., JERNIGAN, J. A., ARAUJO, P. E., APPLEDORF, H., DAVIS, C. G. and DINNING, J. S. Zinc status of elderly black Americans from urban low-income households. Am. J. Clin. Nutr., 33: 1771-1777, 1980.

6. BAILEY, L. B., WAGNER, P. A., CHRISTAKIS, G. J., ARAUJO, P. E., APPLEDORF, H., DAVIS, C. G., MASTERYANNI, J. and DINNING, J. S. Folacin and iron status and hematological findings in predominately black elderly persons from urban low-income households. Am. J. Clin. Nutr., 32: 2346-2353, 1979.

7. YOUNG, V. R. Diet as a modulator of aging and longevity. Federation Proceedings, 38: 1994-2000, 1979.

8. TAMURA, T., SHANE, B., BAER, M. T., KING, J. C., MARGEN, S. and STOKSTAD, E. L. R. Absorption of mono- and polygutamyl folates in zinc-depleted man. Am. J. Clin. Nutr., 31: 1984-1987, 1978.

9. SILINK, M., REDDEL, R., BETHEL, M. and ROWE, P. B. γ-Glutamyl hydrolase (conjugase): Purification and properties of the bovine hepatic enzyme. J. Biol. Chem., 250: 5982-5993, 1975.

10. BAKER, H. FRANK, O., THIND, I., JASLOW, S. and LOURIA, D. Vitamin profiles in elderly persons living at home or in nursing homes, versus profiles in healthy young subjects. J. Am. Geriatrics Soc., 27: 444-450, 1979.

11. Ten-State Nutrition Survey (1968-1970). DHEW Publication No. (HMS) 72-8133, Vol. IV, Washington, D.C., 1973.

12. ABRAHAM, S., LOWENSTEIN, F. W. and JOHNSON, C. L. United States Department of Health Education and Welfare, National Center for Health Statistics: Preliminary Findings of the First Health and Nutritional Examination Survey, United States, 1971-72; Dietary Intake and Biochemical Findings. DHEW Publication No. (HRA) 74-1219-1 Washington D.C., 1974.

13. OWEN, G. M., KRAM, K. M., GARRY, P. J., LOWE, J. E. and RUBIN, H. A. A study of nutritional status of preschool children in the United States. Ped., 53: 597-646, 1974.

14. STRAIN, W. H., PORIES, W. J., FLYNN, A. and HILL, O. A. Jr. Trace element nutriture and metabolism through head hair analysis. In: Trace Substance in Environmental Health--V, edited by HEMPHILL, D. D. University of Missouri, Columbia, pp. 383-397, 1972.

15. SAUBERLICH, H. E., DOWDY, R. P., and SKALA, J. H. Folacin. In: Laboratory Tests for the Assessment of Nutritional Status. CRC Press Inc., Cleveland. pp. 49-60, 1974.

16. SORENSON, R. J., MELBY, C. G., NORD, P. J. and PETERING, H. G. Interferences in the determination of metallic elements in human hair: An evaluation of zinc, copper, lead, and cadmium using atomic absorption spectrophotometry. Archives of Environmental Health, 27: 36-39, 1973.

17. HERBERT, V., BAKER, H., FRANK, O., PASHER, I., SOBOTKA, H., and WASSERMAN, L. R. The measurement of folic acid activity in serum - a diagnostic aid in the differentiation of the megaloblastic anemias. Blood, 15: 228-235, 1960.

18. HOFFBRAND, A. V., NEWCOMBE, B. F. A. and MOLLIN, D. L. Method of assay of red cell folate activity and the value of the assay as a test for folate deficiency. J. Clin. Pathol., 19: 17-28, 1966.

19. BARR, A. J., GOODNIGHT, J. H., SALL, J. P., BLAIR, W. H. and CHILKO, D. M. SAS User's Guide. SAS Institute, Inc., Raleigh, N.C. 1979.

20. DALLMAN, P. R., BARR, G. D., ALLEN G. M., SHINEFIELD, H. R. Hemoglobin concentration in white, black, and oriental children: Is there a need for separate criteria in screening for anemia? Am. J. Clin. Nutr., 31: 377-380, 1978.

NUTRITION RESEARCH, Vol. 1, pp. 571-579, 1981
0271-5317/81/060571-09$02.00/0 Printed in the USA.
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BOUND NIACIN IN SORGHUM AND ITS AVAILABILITY

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ABSTRACT

Two varieties of *Sorghum vulgare*, milo and jowar, have been studied. Samples cooked at neutral pH gave total niacin values of 46 and 29 mg/kg, respectively, on an air-dry basis. In extracts made from these at neutral pH and fractionated after gel-filtration, most of the niacin was in high molecular weight (i.e., bound) forms. In a bioassay for niacin, the boiled milo gave a growth response equivalent to 34% of its total niacin being available to young rats. Sorghum has behaved similarly to other mature cereal grains in these tests of niacin availability.

KEY WORDS: Sorghum, Bound niacin, Nicotinic acid, Gel-filtration, Bioavailability, Pellagra

INTRODUCTION

It has been reported repeatedly by the National Institute of Nutrition (NIN), India, that pellagra is endemic in the Hyderabad region amongst poor people whose staple cereal is a *Sorghum vulgare* known locally as 'jowar' (1). The protein of jowar has a relatively high tryptophan content of 1.2 g/16 g N and the NIN group (2) has also concluded that jowar's *ca.* 30 mg/kg niacin is present in the free (and fully available) form. This would make the occurrence of pellagra doubly puzzling. It would also differentiate jowar from the other mature cereal grains that have been studied (3) and would not agree with another Indian study which indicated that most of the niacin in jowar was in forms unavailable to *Leuconostoc mesenteroides* until released by cooking in alkali (4). We now report a further study of the availability of the niacin in sorghum.

MATERIALS AND METHODS

Preparation of samples

Two varieties of *Sorghum vulgare* were used in the experiments. Whole ground milo of U.S.A. origin was boiled with twice its volume of water (pH 7.0) for 30 minutes. The milo and cooking liquor were freeze-dried together to 2.9% moisture content and ground to pass a 40-mesh sieve. This formed the first test material. Raw jowar obtained from the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Hyderabad, India, was ground to pass a 40-mesh sieve and found to contain 10% moisture. Chapatis were prepared

from part of this sample following the standard procedure used at ICRISAT. 40 ml boiling water was added to 50 g flour, and the dough was kneaded to an even consistency. 20 cm discs (about 1.5 mm thick) were cut out and baked on a flat metal baking sheet at about 260° C. After about 1 minute, water was sprinkled on the top surfaces and after another minute the chapatis were turned over and baked for approximately 2 minutes more until the appearance of light brown spots on the surface indicated that baking was complete. The chapatis were allowed to dry in circulating air to a moisture content of 16%.

Chemical analyses

The samples were analyzed for total nicotinic acid (NA) after alkaline hydrolysis by the AOAC method for cereals (5). Free (NA), nicotinamide (NAM) and bound niacin (BNA) were estimated after extraction and separation using thin-layer chromatography (TLC) and gel-filtration. The extraction using mild conditions to prevent the breakdown of BNA was carried out by homogenizing a known amount of ground sample in 100 ml 50% ethanol in the cold. The homogenate was shaken at room temperature for 30 minutes and centrifuged at 3000 rpm for 15 minutes. The remaining solids were re-extracted in the same way with 50 ml 50% ethanol and re-centrifuged. The solids were further extracted with 50 ml water and centrifuged. The three supernatants were pooled, rotary-evaporated under reduced pressure at 40° C to approximately 5 ml and shaken with 3 ml trichloroethylene. The mixture was then centrifuged at 18,000 rpm at 4° C for 1 hour. The supernatant aqueous layer was removed, evaporated to dryness and taken up in 1 or 2 ml water. The extracts to be alkali-treated were taken up in 1 ml water to which was added 0.5 ml 1.5 M NaOH. This was heated at 100° C for 30 minutes, neutralized with HCl and made up to 2 ml. 1 to 20 µl of the extract together with a standard solution of NA and NAM (containing 0.1 µg/µl were spotted onto TLC plates coated with a 0.25 mm layer of silica gel Gf_{254} (E. Merck, Darmstadt, Germany) previously activated at 110° C for 1 hour. The plates were equilibrated in the vapor of water-saturated *n*-butanol for 1 hour and developed in this solvent by ascending chromatography. They were then dried, exposed to cyanogen bromide vapor for 1 hour and sprayed with a solution of 2% 4-aminobenzoic acid in a 3:1 mix of 0.75 M HCl and 95% ethanol (6).

Since the TLC method is only semi-quantitative, is not very sensitive for NAM, and BNA can only be estimated by difference [total NA - (free NA + NAM)], a method was developed using gel-filtration to separate and quantify NA, NAM and BNA in food extracts directly. Gel-filtration separates compounds in solution according to their molecular sizes. Molecular weights above the exclusion limits do not penetrate the swollen gel and are eluted first. Smaller molecules are fractionated and eluted in order of decreasing molecular size (7).

The best separation of BNA, NA and NAM was obtained using swollen Sephadex G-10 (Pharmacia Fine Chemicals AB, Uppsala, Sweden) poured into a 0.9 cm × 2 m column and equilibrated with 0.1 M sodium phosphate buffer, pH 7.0. For reference purposes, NA (0.1 mg), NAM (0.1 mg) and 4 nCi ^3H-H_2O in 1 ml water containing 2% (w/v) Blue Dextran was loaded onto the column and eluted with the phosphate buffer at a flow rate of 30 ml/hr. Tritiated water was loaded to determine the elution volume of a solute penetrating the gel particles without interacting with them and Blue Dextran to determine the elution volume (void volume) of a compound excluded from the gel. 1 ml of each extract was loaded together with either 20 mg Blue Dextran or 0.1 ml (4 nCi) ^3H-H_2O as reference markers. 5 or 10 ml fractions were collected and each analyzed for NA and NAM. For the reference samples, this was done by measuring the absorbance at 261 nm; Blue Dextran also absorbs at this wavelength. In the case of the test materials, each fraction was hydrolyzed in 0.5 M NaOH at 100° C for 30 minutes, then

neutralized with 6 M HCl and 1 ml phosphate buffer (300 g/liter $Na_2HPO_4 \cdot 7\ H_2O$ + 50 g/liter KH_2PO_4, pH 7). The NA of each fraction was measured using the cyanogen bromide - sulphanilate reaction already used for the estimation of total NA in the test materials (5). ^3H-H_2O was determined by mixing 0.5 ml of each fraction with 1.5 ml scintillation fluid and counting for 10 minutes in a Beckman CP 100 counter.

Tryptophan was measured by a colorimetric method (8), nitrogen by the AOAC Kjeldahl method (9) and N^1-methylnicotinamide (NMN) was estimated fluorimetrically (10).

Bioassay

The bioavailability of niacin was estimated using a rat growth assay (3, 11). Weanling male Wistar rats were placed on a niacin-free, low-tryptophan basal diet for 4 days. They were then allocated to the test diets in a random block design, 2 rats per cage, 4 cages per treatment. A standard response curve was obtained by adding increasing amounts of NA to the basal diet. A test response curve was obtained by adding the test food to the basal diet at 2 levels, with adjustments to ensure that the test diets had the same content of total N and of each essential amino acid as the basal diet. The mean tryptophan value obtained for the boiled milo sample by 4 chemical analyses was 0.97 ± 0.01 g/16 g N; it contained 10.5% crude protein (N × 6.25). In a further, unpublished study, the tryptophan in the milo was found to be fully available to rats (12). Literature values (13) were used to estimate the contents of the other essential amino acids in the milo. The standard and test responses were checked for linearity and a common origin, and the niacin potency of the test food relative to the standard was determined by the slope-ratio procedure (14).

At the end of the 18-day experimental period, 4 rats on the NA-free basal diet and 6 rats from each of the milo diets were injected intraperitoneally with 2.5 mg NAM. Together with the remaining rats in those groups they were maintained on the same diets for a further 4 days. This was done to ensure that the rats on the milo diet could respond to niacin.

RESULTS

Chemical analyses

The chemical results are summarized in Table 1. The values for free NA + NAM and for BNA were those obtained by gel-filtration. Inspection of the thin-layer chromatograms agreed with these in showing little or no free NA in the samples other than the alkali-treated extract. The last sample gave an estimate of 20 mg/kg free NA + NAM which was close to the determined value for gel-filtration.

The results are shown graphically on Figure 1, a redrawn TLC plate. As little as 1 mg NA and 10 mg NAM per kg starting material can be detected by this method. BNA does not migrate from the origin and although it gives the characteristic orange color with cyanogen bromide and 4-aminobenzoic acid, mole for mole it is of lower intensity. NA added to the extract prior to spotting coeluted with standard NA, precluding the possibility that a factor present in the extract could prevent the migration of any free NA in sorghum.

When a mixture of NA, NAM, Blue Dextran and tritiated water was loaded onto the column (Figure 2), as expected, Blue Dextran was eluted with the void volume.

TABLE 1

Total NA, Free NA + NAM, and BNA Content of Sorghum[a]

	Total NA	Free NA + NAM	BNA
Boiled milo	45.5 ± 0.4 (4)	1.6 ± 0.8 (3)	35.1 ± 1.6 (3)
Raw jowar	28.6 ± 0.7 (4)	<1 (2)	19.8 ± 0.6 (2)
Cooked jowar	26.3 ± 0.2 (4)	0.6 ± 0.1 (3)	22.3 ± 1.8 (3)
Cooked jowar with extract alkali-treated	---	21.1 ± 1.4 (3)	1.5 ± 0.7 (3)

[a] mg/kg; mean values ± SE, with number of replicates in parentheses.

NA was retained slightly with respect to the tritiated water and well separated from NAM which was eluted last. The retention of NAM is probably due to interactions with charged groups in the gel. As is also shown on Figure 2, most of the NA in cooked jowar and boiled milo was contained in high molecular weight fractions largely excluded from the gel. This was also the case for raw jowar and in all of these samples, less than 2 mg/kg NA and trace amounts of NAM were detected. In contrast, alkali treatment released the NA of jowar from its bound forms. As little as 0.75 mg/kg BNA, NA or NAM in the starting material could be detected by this method without need of pooling and further concentrating the fractions. Difficulties were encountered obtaining a constant blank value during the total NA assay procedure. This is reflected particularly in the imprecision of the low values obtained. In no way however does this affect the findings that the niacin in sorghum is predominantly bound.

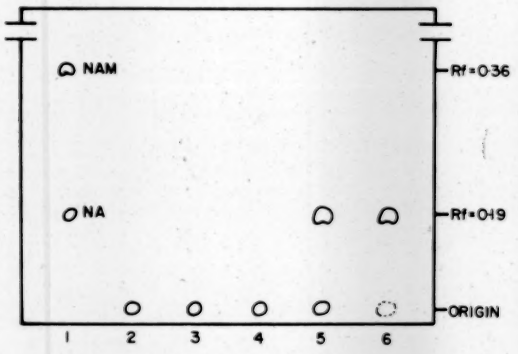

FIG. 1

Thin-layer chromatograms of sorghum extracts and standards: 1 (NA + NAM), 2 (boiled milo), 3 (raw jowar), 4 (cooked jowar), 5 (cooked jowar + NA), 6 (cooked jowar extracts, alkali-treated).

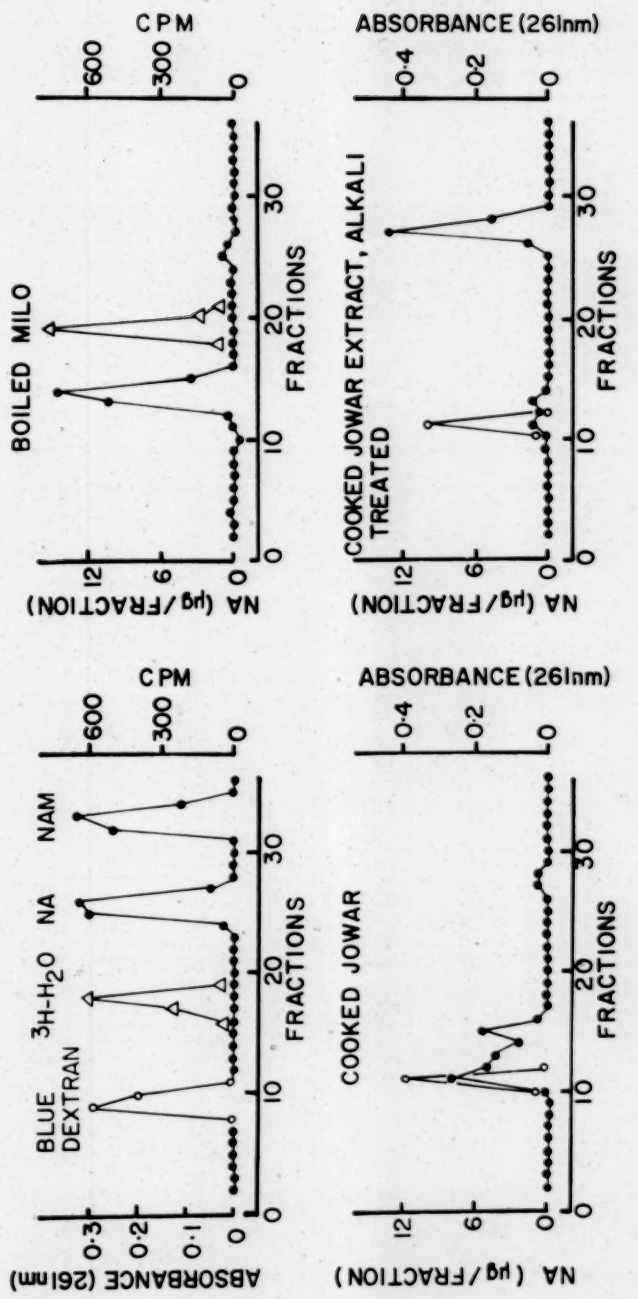

FIG. 2

Elution patterns when standard mixtures or sorghum extracts were eluted from Sephadex G-10 columns. Absorbances for Blue Dextran (o--o) and NA (●--●) and cpm for ^3H-H_2O (Δ--Δ).

Bioassay

The mean growth rates on the different diets are shown in Figure 3. It is seen that the diets containing milo supported rapid growth after the rats had been injected with NAM.

The results after 14, 16 and 18 days on the test diets were analysed separately. The precision of the assay was measured by λ, the ratio of the pooled standard error of the response to an individual diet to the mean increment of response per unit of supplementary NA added to the diet (15). λ was minimal (i.e., preferable) after 16 days and the results over this period, together with the NMN excretion over the two following days are set out in Table 2.

The estimated potency of the milo as estimated from weight gains was 15 ± 3.2 mg/kg, and from FCE was 16 ± 4.5 mg/kg. From the NMN values, the best estimate of potency was approximately 10 mg/kg, but these calculations had an unsatisfactorily high λ value so that it cannot be considered a quantitative estimate. The values from growth and FCE correspond to 33 and 35%, respectively, of the total niacin value obtained by chemical analysis.

FIG. 3

Rat growth curves. Basal diet (o--o), basal supplemented with NA, 4 mg/kg (Δ--Δ), 8 mg/kg (□--□) and 12 mg/kg (▽--▽) or with boiled milo, 84 g/kg (●--●) and 168 g/kg (▲--▲). Final period after injection of 2.5 mg NAM (---).

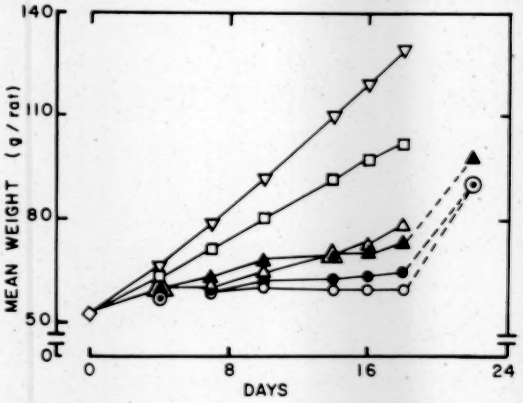

TABLE 2

Weight Gain and FCE of the Rats Over 16 Days and NMN Excretion Over Days 16-18

Dietary supplement	Weight gain (g/rat)	FCE (g gain/g food)	NMN (μg/rat/24 hr)
None	8	0.12	6
Nicotinic acid (4 mg/kg)	20	0.26	9
Nicotinic acid (8 mg/kg)	46	0.36	11
Nicotinic acid (12 mg/kg)	66	0.39	20
Boiled milo (84 g/kg)	10	0.14	6
Boiled milo (168 g/kg)	19	0.22	8
(SE of a treatment mean)	(± 2.3)	(± 0.020)	(± 2.1)

DISCUSSION

The niacin in our sorghum samples was present in chemically bound form(s) which were freed by alkali treatment but not by cooking at neutral pH. This is consistent with results obtained from other mature cereal grains such as corn, wheat, rice, and barley (3, 6, 16) and with findings for jowar itself (4). The finding of some 30-35% availability for rats of the niacin in boiled milo is also in agreement with values for other grains (3). We did not possess sufficient jowar to carry out a bioassay but we have no reason to believe that its bound niacin would behave differently from that in other varieties of sorghum.

The work of Belavady and Gopalan (2) from which they concluded that the niacin in jowar was essentially all in the free form began with extraction of the sample in dilute acid. This procedure had been used by Kodicek and colleagues (17) in their early work but they warned that concentration of these extracts could result in sufficient acidity to hydrolyze the BNA and later omitted the acid from the extraction procedure (6). The jowar extracts were then run on paper chromatograms and judged by eye. Thin-layer chromatograms are less subject to interference and the most recent gel-filtration technique allows subsequent analysis of each fraction. There are grounds, therefore, for thinking that the contrary findings in this study could have been due to the analytical problems at that time rather than to true differences in starting material from those that we used.

Belavady and Gopalan (2) also carried out feeding trials with both rats and dogs from which they concluded that the niacin in jowar was largely available to these species. Inspection of the results from the first rat experiment indicates that the rats all grew at similar rates even though the niacin levels in the diets varied from 8 to 42 mg/kg. The tryptophan levels were apparently too high to allow sensitive conditions for a niacin assay. The second experiment with a low-protein (7%) diet allowed little growth in any diet, even with high niacin supplementation. The same workers reported an experiment with 12 puppies comparing jowar with lime-treated jowar. Eight received very-low-protein diets which did not support growth. The two dogs receiving 8% casein and 80% jowar grew slowly but fairly steadily over a period of 40 weeks. Another two receiving the same diet but with lime-treated jowar showed loss of appetite and some indication of niacin deficiency after 200 days. One died after 260 days and the other showed improvement after injections of nicotinic acid. The lime-treatment certainly appeared to have lowered the value of the jowar rather than to have improved it. McDaniel and Hundley (18) who studied the improvement of corn for dogs by alkali treatment reported that they only obtained the effect when the washing liquors were retained. In the experiment with jowar, the liquors had been discarded but analyses indicated a 60-70% retention of total nicotinic acid. One would still have expected a net improvement if the original niacin had been about 35% available and the alkali-cooking had left the tryptophan undamaged.

It does not seem possible, therefore, to reconcile all the results from various laboratories. However, if the agreed niacin value of about 30 mg/kg for jowar were all available, it would mean that people in the Hyderabad area eating a diet containing 500 g/day (1) would be receiving 15 mg available niacin from this source, which is in excess of the daily requirements (19, 20). If this were the case, there would apparently be no need for an alternative source of niacin from the metabolism of tryptophan. Yet the NIN workers (1) have hypothesized that it is the change in tryptophan metabolism, as a result of the high leucine content of the jowar diets, that is responsible for the pellagra in that area. Such an effect of leucine has not been confirmed in some other studies (21-26). We now know that the 'jowar' pellagrins are also typically deficient in

pyridoxine (27) which could provide another explanation for impaired conversion of tryptophan to niacin. But, to repeat the point, interference with the tryptophan-to-niacin pathway by any mechanism could, it seems, only play a role if the niacin provided by the jowar is largely unavailable.

ACKNOWLEDGEMENTS

We thank Dr. R. Jambunathan, ICRISAT, Hyderabad, India, for supplying the jowar material and the U.S. Department of Agriculture for a grant (5901-0410-9-0330-0) in partial support of this study.

REFERENCES

1. GOPALAN, C. and JAYA RAO, K.S. Pellagra and amino acid imbalance. Vitamins and Hormones 33: 505-527, 1975.

2. BELAVADY, B. and GOPALAN, C. Availability of nicotinic acid in jowar (*Sorghum vulgare*). Indian J. Biochem. 3: 44-47, 1966.

3. CARTER, E.G.A. and CARPENTER, K.J. The availability of niacin in foods. Fed. Proc. 39: 557, 1980.

4. GHOSH, H.P., SARKAR, P.K. and GUHA, B.C. Distribution of the bound form of nicotinic acid in natural materials. J. Nutr. 79: 451-453, 1963.

5. AOAC (Association of Official Analytical Chemists) Official Methods of Analysis, 13th ed. AOAC, Washington, D.C., 1980, pp. 743-746.

6. KODICEK, E. and WILSON, P.W. The availability of bound nicotinic acid to the rat. I. The effect of lime-water treatment of maize and subsequent baking into tortilla. Br. J. Nutr. 13: 418-430, 1959.

7. GELOTTE, B. Studies on gel filtration. Sorption properties of the bed material sephadex. J. Chromatog. 3: 330-342, 1960.

8. MILLER, E.L. Determination of the tryptophan content of feedingstuffs with particular reference to cereals. J. Sci. Fd. Agric. 18: 381-386, 1967.

9. AOAC (Association of Official Analytical Chemists) Official Methods of Analysis, 13th ed. AOAC, Washington, D.C., 1980, pp. 14-15.

10. CARPENTER, K.J. and KODICEK, E. The fluorimetric estimation of N^1-methylnicotinamide and its differentiation from coenzyme I. Biochem. J. 46: 421-426, 1950.

11. CARTER, E.G.A. The Evaluation of Foods as a Source of Niacin. Ph.D. thesis, University of Cambridge, England, 1980.

12. CARTER, E.G.A. and CARPENTER, K.J. Unpublished results.

13. F.A.O. Amino Acid Analysis of Foods and Biological Data on Proteins. Food and Agriculture Organization, Rome, 1970.

14. FINNEY, D.J. Statistical Methods in Biological Assays, 2nd ed. Charles Griffin and Co. Ltd., London, 1964.

15. CARPENTER, K.J., McDONALD, I. and MILLER, W.S. Protein quality of feeding-stuffs. 5. Collaborative studies on the biological assay of available methionine using chicks. Br. J. Nutr. 27: 7-17, 1972.

16. CHAUDHURI, D.K. and KODICEK, E. The availability of bound nicotinic acid to the rat. 4. The effect of treating wheat, rice and barley brans and a purified preparation of bound nicotinic acid with sodium hydroxide. Br. J. Nutr. 14: 35-42, 1960.

17. KODICEK, E., BRAUDE, R., KON, S.K. and MITCHELL, K.G. The effect of alkaline hydrolysis of maize on the availability of its nicotinic acid to the pig. Br. J. Nutr. 10: 51-57, 1956.

18. McDANIEL, E.G. and HUNDLEY, J.M. Alkali-treated corn and niacin deficiency. Fed. Proc. 17: 484, 1958.

19. GOLDSMITH, G.A., ROSENTHAL, H.L., GIBBENS, J. and UNGLAUB, W.G. Studies of niacin requirement in man. II. Requirement on wheat and corn diets low in tryptophan. J. Nutr. 56: 371-386, 1955.

20. HORWITT, M.K., HARVEY, C.C., ROTHWELL, W.S., CUTLER, J.L. and HAFFRON, D. Tryptophan-niacin relationships in man. Studies with diets deficient in riboflavin and niacin together with observations on the excretion of nitrogen and niacin metabolites. J. Nutr. 60(Suppl. 1): 1, 1956.

21. TRUSWELL, A.S. Effect of leucine on N^1-methyl nicotinamide excretion in human adults. Proc. Nutr. Soc. South Afr. 3: 72, 1962.

22. NAKAGAWA, I., OHGURI, S., SASAKI, A., KAJIMOTO, M., SASAKI, M. and TAKAHASHI, T. Effects of excess intake of leucine and valine deficiency on tryptophan and niacin metabolism in humans. J. Nutr. 105: 1241-1252, 1975.

23. NAKAGAWA, I. and SASAKI, A. Effect of an excess intake of leucine, with and without additions of vitamin B_6 and/or niacin, on tryptophan and niacin metabolism in rats. J. Nutr. Sci. Vitaminol. 23: 535-548, 1977.

24. MANSON, J.A. and CARPENTER, K.J. The effect of a high level of dietary leucine on the niacin status of chicks and rats. J. Nutr. 108: 1883-1888, 1978a.

25. MANSON, J.A. and CARPENTER, K.J. The effect of a high level of dietary leucine on the niacin status of dogs. J. Nutr. 108: 1889-1898, 1978b.

26. PATTERSON, J.I., BROWN, R.R., LINKSWILER, H. and HARPER, A.E. Excretion of tryptophan-niacin metabolites by young men: Effects of tryptophan, leucine, and vitamin B_6 intakes. Am. J. Clin. Nutr. 33: 2157-2167, 1980.

27. BAPURAO, S. and KRISHNASWAMY, K. Vitamin B_6 nutritional status of pellagrins and their leucine tolerance. Am. J. Clin. Nutr. 31: 819-824, 1978.

NUTRITION RESEARCH, Vol. 1, pp. 581-587, 1981
0271-5317/81/060581-07$02.00/0 Printed in the USA.
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PROTEIN-INDUCED CALCIURIA IN DIABETIC RATS

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ABSTRACT

To test a proposal that the hypercalciuria induced by excess dietary protein is due to stimulation of insulin secretion, the effects on urinary calcium of feeding a 15% protein versus a 35% protein diet to alloxan-diabetic adult rats were compared. Although diabetes was associated with an increase in urinary calcium, a further increase in calcium, as well as sulfate, excretion was induced by feeding the high protein diet. The results support the conclusion reached in an earlier study that the hypercalciuria of chronic high protein feeding is due mainly to an increase in endogenous acid production arising from the oxidation of excess dietary sulfur amino acids.

Key words: protein, calciuria, diabetes, sulfate, sulfur amino acids.

INTRODUCTION

The increase in urinary calcium in animals and human subjects fed high protein diets (1-3) is the result primarily of a decrease in the fractional reabsorption of this element from the renal tubules (4,5). Hypercalciuria has been attributed mainly to an increase in endogenous acid production arising from the catabolism of excess sulfur amino acids (5,6). High protein diets induce increases in calcium excretion that are proportional to their sulfur content (5).

Recently, an alternative explanation of the hypercalciuria associated with high protein intakes has been proposed, viz., that it is a consequence of the increased circulating insulin level (7) which follows consumption of a protein-rich meal (8). Insulin infusion produces an increase in urinary calcium in human subjects (9) and rats (10), though reportedly not in dogs (11). It is uncertain, however, whether acute insulin-induced changes in calcium excretion are relevant to the chronic hypercalciuria of long-term high protein nutriture.

To test the hypothesis that insulin may be responsible for the hypercalciuria produced by feeding high protein diets, urinary calcium excretion was compared in rats made diabetic by alloxan administration and fed diets containing high versus normal levels of protein.

METHODS

Experiment 1. Male Wistar rats (Woodlyn Laboratories, Guelph, Ontario) weighing approximately 300 g were housed individually in wire-bottom metabolism cages. Food and distilled water were provided ad libitum. Lighting was regulated to give a 12 hr light, 12 hr dark cycle.

The animals were divided into two control (C) groups and two diabetic (D) groups. One control and one diabetic group were fed a normal (15%) protein

diet and the other two groups were fed a high (35%) protein diet (Table 1). Diabetes was produced by repeated intraperitoneal injections of alloxan (Eastman Organic Chemicals, Rochester, NY) dissolved in saline. Each rat received a total dose of 180 mg per kg body weight. All rats were fed the normal protein (NP) diet during the 11-day period of alloxan administration, then one control and one diabetic group were switched to the high protein diet (C-HP and D-HP respectively).

Twenty-four hr urine collections were made on days 2, 4, 10 and 16. Urine was collected in calibrated centrifuge tubes containing 0.5 ml 6N HCl. Urine was analyzed for calcium fluorimetrically (Calcium Analyzer Model 940, Corning Glass Works, Corning, NY), sulfate (12) and glucose (13). The animals were weighed weekly and food intake was recorded during the collection periods.

Experiment 2. The protocol of Experiment 1 was followed with minor changes. Rats weighing approximately 260 g were used and the D-NP and D-HP groups were injected with 90 mg alloxan in divided doses over a 28-day period. Urine was collected on days 2, 4, 8, 10, 15 and 20 post-injection.

Statistical evaluation of the treatment effects was carried out using a one-way analysis of variance. Data are given as $\bar{x}$±SEM.

RESULTS

The diabetic animals excreted approximately 40 times more glucose than the controls (average excretion 2.5 g per 24 hr). In addition, the alloxan-treated groups in both experiments exhibited weight loss (Figure 1) and polyuria (Tables 2 and 3) characteristic of diabetes (14).

TABLE 1

Composition of Diets[1,2]

Ingredient	Normal protein (NP)	High protein (HP)
	g/kg	
Soy protein	150.00	150.00
Lactalbumin	--	201.00
DL-Methionine	2.00	2.00
Lard	150.00	150.00
Corn oil	50.00	50.00
Cellulose	25.00	25.00
Choline chloride	1.00	1.00
Vitamin mix	5.00	5.00
Mineral mix	14.72	14.72
$CaCO_3$	9.72	10.67
$Ca(H_2PO_4)_2 \cdot H_2O$	10.89	7.84
Glucose	581.67	382.77

[1]Details of the diet have been given previously (5).
[2]Both diets contained equal concentrations of Ca (0.6%) and P (0.3%).

TABLE 2

Data for Urinary Sulfate and Calcium and for Urine Volume of Rats in Experiment 1

	CONTROL		DIABETIC	
	Normal protein	High protein	Normal protein	High protein
N	11	8	8	6
VOLUME		ml/24 hr		
Day 2	6.3±0.8	8.9±0.5*	43.4± 7.2	37.8± 5.3
4	5.1±0.9	11.6±1.4*	49.8±11.7	57.8± 5.9
10	5.5±1.1	11.9±0.7*	54.8±15.0	81.5±14.6
SULFATE		mEq/24 hr		
Day 2	0.69±0.05	1.11±0.09*	0.46±0.06	1.04±0.24**
4	0.63±0.07	0.94±0.06*	0.44±0.11	1.49±0.22**
10	0.63±0.09	1.45±0.08**	1.46±0.22	2.23±0.40*
CALCIUM		mg/24 hr		
Day 2	0.50±0.08	3.01±0.46**	2.47±0.40	4.23±0.73*
4	0.68±0.06	2.36±0.26**	3.42±1.00	7.50±1.10**
10	0.58±0.08	1.25±0.24**	3.58±0.60	12.02±2.45*
16	0.43±0.05	1.01±0.20**	4.59±1.40	7.54±0.91

*P<0.05, **P<0.01 with respect to value for respective normal protein group.

TABLE 3

Data for Urine Volume and Urinary Sulfate of Rats in Experiment 2

	CONTROL		DIABETIC	
	Normal protein	High protein	Normal protein	High protein
VOLUME		ml/24 hr		
Day 2	7.8±1.8	9.7±1.5	193±11	197±11
4	8.8±3.1	15.2±4.4	200±12	192±11
8	9.0±3.7	12.4±3.5	196±12	193±11
10	6.8±1.7	15.9±5.6	178± 9†	181± 8†
15	8.1±1.9	20.6±3.9*	149±20†	136±13†
20	8.9±2.6	16.2±3.4	141±18†	144±15†
SULFATE		mEq/24 hr		
Day 2	0.67±0.07	1.26±0.09**	--	3.06±0.45
4	0.78±0.08	1.28±0.12**	1.59±0.24	5.98±0.70**
8	0.83±0.06	1.40±0.12**	1.70±0.24	3.41±0.48**
10	0.84±0.16	1.74±0.31**	1.19±0.26	3.08±0.29**
15	0.64±0.03	1.62±0.11**	--	4.57±0.54
20	0.49±0.09	1.89±0.16**	1.16±0.14	3.05±0.45**

*P<0.05, **P<0.01 with respect to value for respective normal protein group. N=9 except †N=8.

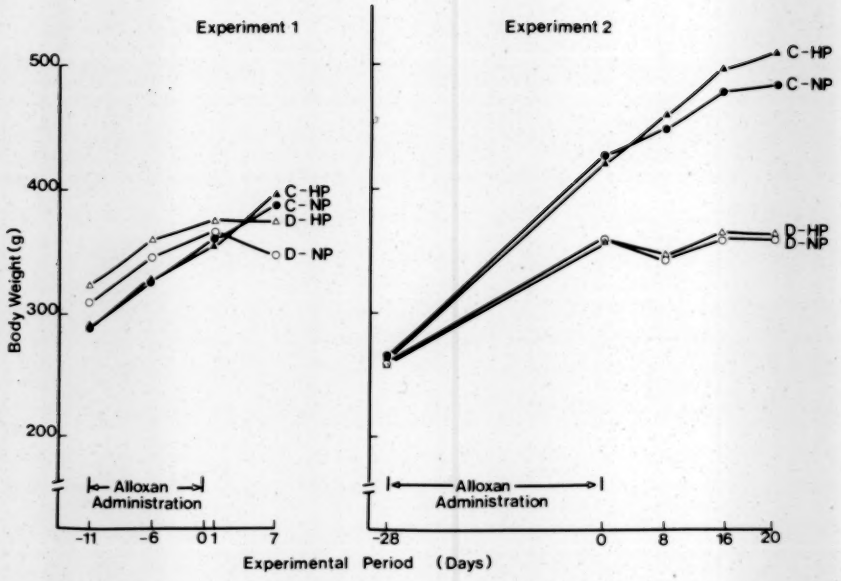

FIG. 1

Effect of alloxan administration and dietary protein on body weight. C-NP=control-normal protein; C-HP=control-high protein; D-NP=diabetic-normal protein; D-HP=diabetic-high protein. During alloxan administration all animals were fed the normal protein diet.

High protein feeding had no effect on the body weight of either the control or diabetic rats (Figure 1). At the end of each experiment, the diabetic rats were consuming as much food as their respective controls, but were gaining less weight.

The high protein diet significantly increased urine volume and sulfate excretion in the control animals of both Experiment 1 (Table 2) and Experiment 2 (Table 3). In the diabetic rats, high protein feeding caused an increase in urine sulfate but not in urine volume.

Both the control and the diabetic animals fed the high protein diet exhibited an increase in urinary calcium relative to that of the corresponding animals fed the normal protein diet (Table 2, Figure 2). The peak of calcium excretion in the controls was seen at day 2 in both experiments. In Experiment 1 the diabetic animals exhibited a significant protein-induced hypercalciuria on days 2, 4 and 10 (Table 2). At day 16 and beyond (data not shown) the difference between the two diabetic groups was not significant ($P>0.05$). Similarly, the calciuretic response of the diabetic animals of Experiment 2 was greatest during the first week of high protein feeding (Figure 2).

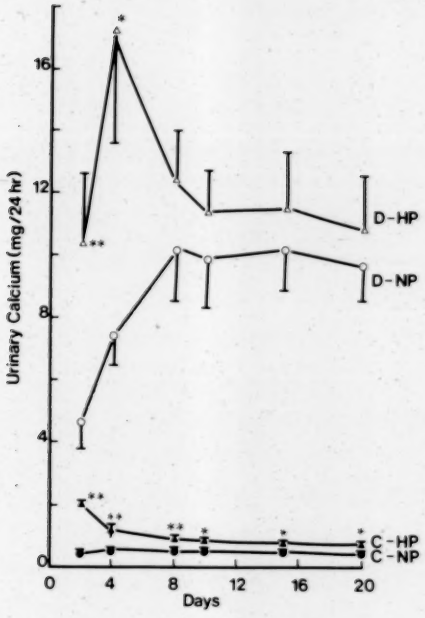

FIG. 2

Effect of dietary protein on urinary calcium in diabetic and control rats of Experiment 2. See caption for FIG. 1.
**P<0.01. *P<0.05.

DISCUSSION

This study demonstrates that the calciuretic response to a high protein diet is present in animals lacking insulin secretory ability. The response was more pronounced during the first 10 days of high protein feeding, whereafter smaller differences in mean calcium excretion rates were rendered non-significant by the variability in calcium excretion in uncontrolled diabetes. As reported by other investigators (14,15) urinary calcium was markedly affected by insulin status.

The diabetic rat has a diminished capacity for intestinal absorption of calcium (14,16) and develops fragile bones (15). It was recently proposed (10) that the presence of glucose in the distal tubular fluid prevents calcium reabsorption in that segment of the nephron. The glucose-mediated increase in calcium excretion occurs in the absence of parathyroid hormone (10). However, the mechanism by which insulin affects urinary calcium is not fully understood.

Following a protein meal, there is an increase in both urinary calcium and serum insulin (7). The rise in urinary calcium occurs one-half hour after the rise in serum insulin, suggesting that insulin does not act directly on the renal tubules. Infusion of insulin into human subjects also does not result in an immediate renal response (9). In animals, an effect of insulin on the renal handling of calcium has been reported to be absent (11) or to be explainable in terms of glucose changes alone (10).

The renal response to a protein load administered as a single meal may not be the same as the response to chronic consumption of a high protein diet. Although an increase in net acid excretion after a high protein meal was not detectable (17), it is well-established that acid excretion is elevated in human subjects consuming high protein diets (6). Following ingestion of a high protein meal, the immediate post-prandial period is marked by a "gastric alkaline tide" (18). With gastric stimulation, there is a transient increase in serum calcium ion concentration and in serum pH (18). The rise in pH may lead to an increase in sulfate in the serum and extracellular fluid, and to a delay in its excretion in the urine.

REFERENCES

1. BELL, R.R., ENGLEMAN, D.T., SIE, T.-L. and DRAPER, H.H. Effect of a high protein intake on calcium metabolism in the rat. J. Nutr., 105: 475-483, 1975.

2. MARGEN, S., CHU, J-Y., KAUFMANN, N.A. and CALLOWAY, D.H. Studies in calcium metabolism. I. The calciuretic effect of dietary protein. Amer. J. Clin. Nutr., 27: 584-589, 1974.

3. KIM, Y. and LINKSWILER, H.M. Effect of protein intake on calcium metabolism and on parathyroid and renal function in the adult human male. J. Nutr., 109: 1399-1404, 1979.

4. ALLEN, L.H., BARTLETT, R.S. and BLOCK, G.D. Reduction of renal calcium reabsorption in man by consumption of dietary protein. J. Nutr., 109: 1345-1350, 1979.

5. WHITING, S.J. and DRAPER, H.H. The role of sulfate in the calciuria of high protein diets in adult rats. J. Nutr., 110: 212-222, 1980.

6. CHAN, J.C.M. The influence of dietary intake on endogenous acid production. Nutr. Metabol., 16: 1-9, 1974.

7. ALLEN, L.H., BLOCK, G.D., WOOD, R.J. and BRYCE, G.F. The role of insulin and parathyroid hormone in the protein-induced calciuria of man. Nutr. Res., 1: 3-11, 1981.

8. FAJANS, S.S., FLOYD, J.C., KNOPF, R.F. and CONN, J.W. Effect of amino acids and proteins on insulin secretion in man. Recent Prog. Horm. Res., 23: 617-662, 1967.

9. DeFRONZO, R.A., COOKE, C.R., ANDRES, R., FALOONA, G.R. and DAVIS, P.J. The effect of insulin on the renal handling of sodium, potassium, calcium and phosphate in man. J. Clin. Invest., 55: 845-855, 1975.

10. ROY, D.R. and SEELY, J.F. Effect of glucose on renal excretion of electrolytes in the rat. Am. J. Physiol., 240: F17-F24, 1981.

11. DeFRONZO, R.A., GOLDBERG, M. and AGUS, Z.S. The effects of glucose and insulin on renal electrolyte transport. J. Clin. Invest., 58: 83-90, 1976.

12. MA, R.S.W. and CHAN, J.C.M. Endogenous sulfuric acid production: a method of measurement by extrapolation. Clin. Biochem., 6: 82-87, 1973.

13. SOMOGYI, M. A rapid method for the estimation of urine sugar. J. Lab. Clin. Med., 26: 1220-1223, 1941.

14. CHARLES, M.A., TIRUNAGURU, P., ZOLOCK, D.J. and MORRISSEY, R.L. Duodenal calcium transport and calcium binding protein levels in experimental diabetes mellitus. Mineral Electrolyte Metab., 5: 15-22, 1981.

15. DIXIT, P.K. and EKSTROM, R.A. Decreased breaking strength of diabetic rat bone and its improvement by insulin treatment. Calcif. Tissue Int., 32: 195-199, 1980.

16. SCHNEIDER, L.E. and SCHEDL, H.P. Effects of Solanum malacoxylon on duodenal calcium binding protein in the diabetic rat. Endocrinology, 100: 928-933, 1977.

17. BLOCK, G.D., WOOD, R.J. and ALLEN, L.H. A comparison of the effects of feeding sulfur amino acids and protein on urine calcium in man. Amer. J. Clin. Nutr., 33: 2128-2136, 1980.

18. HUGHES, W., COHEN, S., ARVAN, D. and SEAMONDS, B. The effect of the alkaline tide on serum-ionized calcium concentration in man. Digestion, 15: 175-181, 1977.

NUTRITION RESEARCH, Vol. 1, pp. 589-599, 1981
0271-5317/81/060589-11$02.00/0 Printed in the USA.
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ZINC-INDUCED ANEMIA IN YOUNG JAPANESE QUAIL AMELIORATED BY SUPPLEMENTAL COPPER AND IRON

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ABSTRACT

Studies were conducted to examine whether anemia induced in newly hatched Japanese quail by moderate dietary Zn supplements could be corrected by supplemental Fe, Cu, or Mg by 14 d of age. Low hemoglobin (Hb) was partially alleviated by increased levels of Fe or Cu, but normal Hb was obtained only with Fe plus Cu. Fe and Cu concentrations in the liver were decreased at 7 d, prior to the development of anemia by 13 d. Additional Mg had no effect on low Hb caused by Zn. Copper supplementation partially alleviated the growth depression and entirely prevented the lack of feather pigmentation that resulted from Zn toxicosis. Neither Fe nor Mg affected either of these parameters. These data indicate that both Fe and Cu status of an individual may be compromised by dietary Zn supplementation.

KEY WORDS: Zinc, Copper, Iron, Anemia, Diet, Mineral Supplements

INTRODUCTION

The increased consumption of zinc as supplements in concentrated form and from individual foods fortified with zinc, as well as therapeutic doses for medical purposes, has created concern about the potential adverse health effects of moderate levels of excess dietary zinc. Several investigators have shown that high levels of dietary zinc cause a microcytic, hypochromic anemia in experimental animals (1-4). Settlemire and Matrone (5,6) and Coleman and Matrone (7) showed that rats fed 7500 ppm zinc had deformed, fragile erythrocytes, and livers with reduced amounts of ferritin and lower concentration of iron in the ferritin. Cox and Harris (8) noted that in rats fed 4000 ppm dietary zinc, hemosiderin, as well as ferritin, content of the liver was reduced.

Additionally, interference with the normal absorption and/or metabolism of iron and copper have been suggested by reports of reduced liver iron and/or copper concentrations in rats fed dietary zinc ranging from 2000 - 10,000 ppm (3,4,9,10). Cox and Harris (9) stated that the high dietary zinc resulted first in reduced liver iron concentration, followed by reduced liver copper concentration. A dietary level of 4000 ppm zinc fed to pregnant or lactating rats produced changes in the iron and copper concentrations in the tissues of the pups (11,12).

Grant-Frost and Underwood (3) suggested that zinc either reduced the availability of tissue copper or caused a deficiency of another dietary element required for hematopoiesis. Cox and Harris (9) suggested that the decreased concentration of liver iron, not copper, was responsible for the anemia caused by high levels of dietary zinc; in spite of the fact that supplemental iron only partially prevented the anemia, whereas supplemental copper completely

prevented it. However, Magee and Matrone (4) reported that supplements of iron or copper, individually, effected equal magnitudes of increase in the hemoglobin level of rats fed 7500 ppm zinc. Only a combined supplement restored hemoglobin levels to those of the control rats.

In the above studies zinc was fed at relatively high levels, greater than 1000 ppm. Also, dietary levels of other essential minerals were generally in excess of requirement. The most sensitive responses reported to have resulted from high zinc intake were observed in animals receiving minimally adequate levels of copper. In rats fed a diet with only sufficient copper to meet estimated requirements, 300 ppm zinc reduced plasma ceruloplasmin activity, although no change in hemoglobin was observed (13). With 250 ppm supplemental zinc, Hamilton et al. (14) demonstrated a decrease in hemoglobin and hematocrit values as well as a reduction in iron and copper concentrations in the liver of young Japanese quail. Campbell and Mills (15) found that 750 ppm dietary zinc induced a severe copper deficiency in pregnant sheep which was prevented by supplementary copper. With copper-deficient diets 200 ppm zinc caused anemia in chicks which was prevented by the addition of copper (16,17).

In human studies it has been reported that a patient receiving zinc therapy developed symptoms of severe anemia which were corrected by withdrawal of the zinc supplements and administration of oral copper (18). Dialysis anemia has been related to high zinc concentrations in the dialysate (19). Prasad et al. (20) reported the occurrence of copper deficiency (decreased levels of plasma copper and ceruloplasmin) in sickle-cell patients treated with 150 mg zinc per day, which was corrected by copper supplementation. Recently, Abdulla (21) reported that adults given only 42 mg zinc per day had reduced plasma copper after 2 weeks.

The purpose of this study was to determine if the anemia caused by moderate levels of supplemental dietary zinc in young Japanese quail (14) could be corrected by increased dietary levels of iron, copper and/or magnesium, elements required for maintaining normal hemoglobin in the young quail.

EXPERIMENTAL

Newly hatched Japanese quail (Coturnix coturnix japonica) of both sexes from our stock colony were assigned to experimental groups of 10 birds each. The birds were housed in suspended stainless steel cages under conditions that minimized environmental contamination. They received continuous light and adequate heat. Diet and deionized water were available at all times.

The birds were fed purified casein-gelatin diets that were adequate in all nutrients insofar as requirements are known. Zinc, iron, manganese, copper, and magnesium were present in the basal diet at requirement levels only. Details regarding diet composition and preparation are given in Table 1. Supplemental zinc was supplied in the diets as zinc carbonate at levels of 250 and/or 500 ppm. Unless otherwise noted, levels of zinc fed will refer to zinc added as a supplement, in excess of that in the basal diet (20 ppm; see Table 1, footnote 5). Supplemental iron, copper, and magnesium were added as ferrous sulfate, copper sulfate, and magnesium sulfate.

The levels of supplemental zinc, iron, copper, and magnesium used in each experiment are shown in Table 2. The iron and/or copper-deficient diets for experiment 6 were prepared by reduction in the amount of salts added to the basal diet. The birds were fed the experimental diets from hatching to 14 days, except in experiment 1, which was terminated at 7 days.

TABLE 1
Diet Composition[1]

Ingredient	(g/kg)
Casein, vitamin-free[2]	280.0
Gelatin[3]	70.0
L-Arginine hydrochloride, NRC[4]	6.0
Glycine[4]	5.0
DL-Methionine, NRC[4]	6.0
Choline dihydrogen citrate[4]	8.4
Mineral mixtures[5]	58.4
Corn oil[6]	40.0
Glucose monohydrate[7]	526.2

[1] The B vitamins were mixed with 10 g glucose/kg diet and the fat-soluble vitamins were mixed with 5 g corn oil/kg diet. Vitamins were present as follows (mg/kg diet): thiamine-HCl 8, riboflavin 8, D-Ca pantothenate 40, nicotinic acid 100, pyridoxine-HCl 8, folic acid 3, vitamin B_{12} 0.02, biotin 0.6, retinyl acetate 6, DL-α-tocopherol 25, DL-α-tocopheryl acetate 25, menadione 1, vitamin D_3 0.02.
[2] Teklad Test Diets, Madison, WI
[3] Dyna-Gel, Calumet City, IL
[4] Grand Island Biological Co., Grand Island, NY
[5] Except for Mg, the macroelements and I were supplied as follows (g/kg diet): $CaHPO_4$ 28.39, $CaCO_3$ 10, NaCl 4, Na_2HPO_4 7, KCl 7 plus KIO_3 0.0099. Se (0.2 mg) was added as Na_2SeO_3. For other elements the dietary totals by analysis were (ppm): Zn 20, Fe 100, Mn 2.5, Cu 1.5, and Mg 300. Individual mineral powders were prepared from glucose monohydrate and zinc carbonate, ferric citrate, manganese sulfate, copper sulfate, or magnesium sulfate.
[6] Mazola, Best Foods, Englewood Cliffs, NJ
[7] Cerelose, Corn Products, Englewood Cliffs, NJ

Birds were wing-banded at 6 days of age and weighed on days 7 and 14. On day 13 hemoglobin was measured and wing feather pigmentation evaluated as described previously (14).

At the end of experiments 1, 2 and 5 the birds were killed by decapitation and the livers were excised immediately, rinsed in 0.75% saline solution, and blotted dry. The tissues were placed in tightly sealed, preweighed plastic bags. Samples were weighed and stored at -15C until analysis.

The tissues were wet digested in a 5:1 (v/v) mixture of concentrated nitric and 70% perchloric acids. The zinc, iron, manganese, copper, and magnesium contents of the tissues were determined by conventional flame atomic absorption spectrophotometry (Perkin-Elmer Corp., Model No. 5000). To minimize phosphate interference and to control viscosity, the final sample dilutions contained 10% glycerol and 7% perchloric acid by volume (22). Using these procedures to analyze the National Bureau of Standards' Liver Reference Material produced values for these elements within the ranges certified. Liver mineral values are expressed on the basis of fresh weight. Diets and mineral premixes were assayed by the same techniques.

TABLE 2
Supplemental Levels of Zinc, Iron, Copper, and Magnesium used in Experiments 1 through 6[1]

Experiment	Supplement (ppm)			
	Zn	Fe	Cu	Mg
1	0, 250	0	0	0
2	0, 250, 500	0	0	0
3[2]	500	0, 100	0, 1.5	0, 100
4[2]	500	0, 100, 200	0, 1.5, 5.0	0
5	0, 500	0, 100	0, 1.5	0
6[3]	0, 250	deficient, 0	deficient, 0	0

[1] Each experiment utilized a factorial arrangement. The dietary levels of iron, copper, and magnesium were requirement levels except in experiment 6 in which iron and/or copper were deficient in some groups.
[2] This experiment included an additional group with no supplements.
[3] Deficient iron and copper levels were 35 and 1.1 ppm, respectively.

Student's *t* test (23) was used to evaluate the data, except for the white feather scores, which were treated as ordinal-scale data and evaluated using the Wilcoxon Signed Rank Test (24).

RESULTS

There was no significant mortality in any of the experiments. Data from the first two experiments for body weight, hemoglobin, and concentrations of zinc, iron, manganese, copper, and magnesium in the liver are shown in Table 3. All measurements, except concentration of magnesium in the liver, were affected by zinc. Body weight was not significantly depressed except at two weeks with 500 ppm zinc. Hemoglobin was reduced at 7 and 13 d by 500 ppm zinc, but 250 ppm reduced hemoglobin only at 13 d. At one week 250 ppm zinc caused increased zinc and decreased iron, manganese, and copper concentrations in the liver. Iron, manganese, and copper concentrations in the liver also were all decreased by 500 ppm zinc at two weeks, whereas zinc concentrations were increased by both 250 and 500 ppm dietary zinc.

The effects of supplements of iron, copper, and magnesium, singly and in combination, on body weight, feather pigmentation and hemoglobin of birds fed 500 ppm zinc are reported in Table 4. In both experiments body weight was reduced by excess zinc and in all cases, except one, remained significantly depressed if copper was not included in the supplement. With copper supplementation white feather score consistently was improved relative to that of the control. Magnesium supplementation had no effect on hemoglobin level. Single supplements of iron and copper in experiment 3 had no effect. In experiment 4, except for 200 ppm iron, all supplements increased the hemoglobin concentrations relative to that caused by excess zinc alone; however, the single supplements did not increase hemoglobin concentration to equal the control with no excess zinc. In both experiments only the combined supplements of iron and copper resulted in a hemoglobin level equal to the control.

TABLE 3
Effects of Supplemental Dietary Zinc on Body Weight, Hemoglobin, and Mineral Concentrations in the Liver of Japanese Quail at 7 or 14 Days of Age[1]

Zinc (ppm)	Body wt (g)		Hemoglobin (g/dl)		Liver Elements				
	7d	14d	7d	13d	Zn (ppm)	Fe (ppm)	Mn (ppm)	Cu (ppm)	Mg (ppm)
				Experiment 1					
0	21±1.3	-	9.5±0.48	-	19±0.5	155±20.1	1.8±0.10	2.8±0.18	225±5
250	21±0.5	-	10.3±0.24	-	36±5.9a	110±14.6a	1.4±0.16a	2.3±0.12a	226±4
				Experiment 2					
0	22±0.8	45±1.7	10.7±0.41	12.3±0.32	21±1.0	152±20.1	2.0±0.12	4.0±0.16	238±6
250	23±0.6	44±1.0	9.9±0.37	11.2±0.32a	33±3.5A	123±13.7	1.9±0.27	2.4±0.12A	247±5
500	21±0.8	40±1.0a	8.8±0.29A	10.6±0.32A	43±4.6A	75± 9.4A	1.1±0.10A	2.0±0.18A	226±7

[1] Mean values ± S.E. Mean values were significantly different within experiments from the group with no supplemental zinc: a,$P<0.05$, A,$P<0.01$. Mineral values are on the basis of fresh weight. Experiment 1 was terminated at 7 days and experiment 2 at 14 days.

TABLE 4
Effects of Supplemental Iron, Copper and Magnesium on Response of Japanese Quail Fed Excess Dietary Zinc to 14 Days of Age[1]

Supplement (ppm)				Body wt	White feather	Hemoglobin
Zn	Fe	Cu	Mg	(g)	score [2]	(g/dl)
				Experiment 3		
0	0	0.0	0	45±1.4b	0.1±0.07B	12.3±0.17B
500	0	0.0	0	40±0.8a	3.1±0.23A	10.0±0.42A
500	100	0.0	0	40±0.7a	2.8±0.45A	10.7±0.71a
500	0	1.5	0	41±1.4	0.1±0.11B	10.9±0.42a
500	0	0.0	100	42±0.9a	2.4±0.44A	9.5±0.52A
500	100	1.5	0	41±1.7	0.4±0.31B	12.1±0.62b
500	100	0.0	100	37±1.6A	2.7±0.31A	9.5±0.47A
500	0	1.5	100	44±0.7B	0.2±0.12B	10.8±0.38A
500	100	1.5	100	41±1.8	0.0B	11.8±0.42B
				Experiment 4		
0	0	0.0	0	46±1.4B	0.2±0.20B	12.4±0.12B
500	0	0.0	0	37±2.0A	2.2±0.33A	10.1±0.37A
500	100	0.0	0	39±1.8A	2.7±0.15A	11.5±0.38ab
500	200	0.0	0	37±1.9A	2.9±0.28A	10.6±0.43A
500	0	1.5	0	43±0.5b	0.0B	11.4±0.38ab
500	0	5.0	0	43±0.9b	0.0B	11.4±0.28Ab
500	100	1.5	0	43±0.9b	0.1±0.10B	13.2±0.48B
500	100	5.0	0	40±1.6a	0.0B	12.8±0.38B
500	200	1.5	0	45±1.1B	0.4±0.21B	11.6±0.15AB
500	200	5.0	0	46±2.3b	0.0B	12.5±0.40B

[1] Mean values ± S.E. Mean values were significantly different within experiments from the group with no supplemental zinc: a, $P<0.05$, A, $P<0.01$; from the group with only supplemental zinc: b, $P<0.05$, B, $P<0.01$.
[2] Scored on a basis of 0 to 4, with 0 as normal and 4 as the maximal abnormality.

In the absence of excess zinc there were no effects of supplemental iron and/or copper on body weight, feather pigmentation, or hemoglobin (Table 5), except for a small effect of iron on body weight. In the presence of excess zinc, copper had beneficial effects on body weight and feather pigmentation, and the copper and/or iron supplements affected hemoglobin levels, as in experiments 3 and 4. Supplemental iron or copper was reflected in the liver concentration of each element. When the single supplements were fed in the absence of excess zinc, no imbalance or interaction was observed with the other element. Zinc concentration in the liver was increased by supplemental zinc, except with copper supplementation alone. Excess zinc caused a decrease in liver iron, which was alleviated by the supplemental iron; however, iron concentrations of all groups with excess zinc were markedly lower than corresponding groups without added zinc. Liver copper was decreased in the presence of excess zinc. Normal copper concentrations were achieved with copper supplementation.

TABLE 5
Effects of Supplemental Iron and Copper on Body Weight, Feather Pigmentation, Hemoglobin, and Mineral Concentrations in the Liver of Japanese Quail Fed Supplemental Dietary Zinc to 14 Days of Age[1]

Suppl. (ppm) Zn	Fe	Cu	Body wt (g)	White Feather Score[2]	Hemoglobin (g/dl)	Liver Elements (ppm) Zn	Fe	Cu
				Experiment 5				
0	0	0.0	44±1.1	0.0	12.3±0.24	20± 1.0	94± 7.6	3.1±0.13
0	100	0.0	48±1.4a	0.2±0.12	12.6±0.29	20± 1.1	457±40.5A	3.3±0.10
0	0	1.5	44±2.0	0.0	12.4±0.18	24± 3.2	102± 9.1	4.1±0.40a
0	100	1.5	44±0.9	0.0	12.7±0.31	21± 0.5	287±51.9A	5.1±0.34A
500	0	0.0	36±1.2AB	2.9±0.44AB	7.8±0.28AB	44± 7.0ab	58± 5.9AB	1.3±0.12AB
500	100	0.0	37±1.5AB	2.8±0.16AB	10.0±0.53ABC	59±10.1AB	81± 8.8Bc	0.9±0.13ABc
500	0	1.5	43±0.9C	0.9±0.23AbC	10.3±0.52ABC	29± 1.9A	45± 3.7AB	3.1±0.33bC
500	100	1.5	38±1.4AB	0.5±0.27C	12.3±0.45C	42± 5.6AB	95±23.0B	2.9±0.20BC

[1] Mean values ± S.E. Mean values were significantly different from the group with no supplements: a, $P<0.05$, A, $P<0.01$; from the corresponding group with no supplemental zinc: b, $P<0.05$, B, $P<0.01$; from the group with only supplemental zinc: c, $P<0.05$, C, $P<0.01$. Mineral values are on the basis of fresh weight.
[2] Scored on a basis of 0 to 4, with 0 as normal and 4 as the maximal abnormality.

In experiment 6, body weight was decreased by supplemental zinc only when the diet was deficient in copper (Table 6). White feathers were observed in all groups that were either deficient in copper or fed excess zinc. The severity was greatest in those copper-deficient groups that were fed supplemental zinc. All groups that were iron deficient were severely anemic. Marginal dietary copper deficiency did not affect the hemoglobin concentration except in the presence of excess dietary zinc and adequate dietary iron.

DISCUSSION

Although we have shown a significant reduction in hemoglobin with 250 ppm zinc (Table 3; see also 14), 500 ppm was chosen as the level to use for this study because it uniformly produced a very significant but not maximal reduction in the hemoglobin concentration. This provided an intermediate response that could be either ameliorated or exacerbated by dietary treatment.

Stewart and Magee (25) concluded that high levels of zinc have an adverse effect on both absorption and utilization of magnesium. Young Japanese quail respond very sensitively to dietary magnesium deficiency by a decrease in hemoglobin level (26). It was thought that zinc antagonism of magnesium might be involved in the low hemoglobin produced by zinc even though zinc had not decreased magnesium concentrations in the tibia (27). Since magnesium supplementation had no effect on zinc-induced anemia in these experiments, it does not appear that a zinc-magnesium antagonism was involved. Additionally, concentrations of magnesium in the liver could not be related to any changes in hemoglobin levels.

TABLE 6
Effect of Iron and/or Copper Deficiency on Response of Japanese Quail Fed Supplemental Zinc to 14 days of age[1]

Supplemental Zn (ppm)	Dietary Fe (ppm)	Dietary Cu (ppm)	Body wt (g)	White feather score [2]	Hemoglobin (g/dl)
			Experiment 6		
0	35	1.1	41±1.2	1.5±0.34	7.1±0.44
250	35	1.1	33±0.9A	3.0±0.30A	6.5±0.57
0	35	1.5	40±1.4	0.0B	7.6±0.38
250	35	1.5	40±1.6B	1.8±0.33Ab	6.3±0.24A
0	100	1.1	40±1.6	1.2±0.40	12.4±0.32C
250	100	1.1	33±2.0a	3.6±0.40A	10.5±0.79aC
0	100	1.5	45±1.1bcd	0.0bD	12.4±0.18CD
250	100	1.5	42±1.5Bd	1.6±0.41aBd	12.1±0.42bCD

[1] Mean values ± S.E. Mean values were significantly different from the corresponding group with no supplemental zinc: a, $P<0.05$, A, $P<0.01$; from the group receiving 1.1 ppm copper with the same levels of dietary iron and supplemental zinc: b, $P<0.05$, B, $P<0.01$; from the group receiving 35 ppm iron with the same levels of dietary copper and supplemental zinc: c, $P<0.05$; C, $P<0.01$; between the groups receiving 1.1 ppm copper and 35 ppm iron and the groups receiving 1.5 ppm copper and 100 ppm iron with the same level of supplemental zinc: d, $P<0.05$, D, $P<0.01$.

[2] Scored on a basis of 0 to 4, with 0 as normal and 4 as the maximal abnormality.

Several investigators (4,11,13) have shown reduced iron and/or copper concentrations in the livers of rats fed much higher levels of excess zinc (1000 - 7500 ppm) than were fed in these experiments. We also observed decreased levels of iron and copper in the livers of quail fed moderately high zinc (250-500 ppm) (Table 3; see also 14).

The anemia induced by excess zinc was partially alleviated by levels of supplementary iron or copper that maintained normal liver levels of these elements; however, higher levels of either iron or copper offered no further improvement in hemoglobin values. To achieve normal hemoglobin levels with the high-zinc diets, supplementation with both iron and copper was required. These results agree with those previously observed in rats fed much higher levels of zinc: 7500 ppm (4) and 5000 ppm (3), and support the thesis that excess dietary zinc antagonizes both iron and copper. This is in contrast to the observations of Cox and Harris (9) that iron partially alleviated and copper completely alleviated anemia induced by 4000 ppm zinc.

In the presence of excess zinc the fact that the higher supplement of iron, a level above that necessary to give normal liver iron, did not increase the hemoglobin level to that of the control, suggests that another factor(s) required for normal hemoglobin is antagonized by zinc. Campbell and Mills (13) reported a decrease in ceruloplasmin by excess zinc. Although the effect of copper on iron mobilization via ceruloplasmin is well known (28,29) there is still the possibility that copper may have an additional direct and specific effect on the production and development of reticulocytes (30). The data also show that when copper supplementation is adequate to prevent signs of copper deficiency (decreased liver copper and white feathers) and therefore presumably adequate

for iron mobilization, hemoglobin is still less than normal, suggesting a direct antagonism of iron by excess zinc. This has been postulated to be related to iron incorporation and release from ferritin and reduced life span of the red blood cells (6). Although iron had a beneficial effect in partially alleviating the anemia due to excess zinc, it tended to increase the severity of zinc-induced perosis. This probably resulted from further antagonism of manganese. This adverse affect of zinc on bone development will be discussed in a separate paper.

Hamilton et al. (14) reported that when Japanese quail were fed graded levels of excess zinc with two levels of dietary copper (1.1 and 3.6 ppm), the higher level of dietary copper did not improve the hemoglobin and hematocrit values. The manganese level of that diet was 6.8 ppm, as compared to the requirement level of 2.5 ppm present in these studies. It is thought that a manganese antagonism of copper and/or iron might have affected hematopoiesis.

Cox and Harris (9) reported that 4000 ppm excess zinc fed to weanling rats caused a relatively rapid initial decrease of iron concentration in the liver, followed by anemia and later by a decline of copper concentration in the liver. They proposed that the decreased liver iron, but not decreased copper, was responsible for the zinc-induced anemia. We found, however, that both iron and copper concentrations in the liver were reduced at 7 days, prior to the development of anemia at 13 days (Table 3). Hill and Matrone (16) reported that when higher levels of zinc (1000 - 2500 ppm) were fed to chicks, there was an apparent antagonism of iron with decreased hemoglobin, which was partially corrected by copper. At lower levels of excess zinc (100 - 200 ppm), copper supplementation resulted in hemoglobin levels equal to non-zinc supplemented controls. However, these chicks were copper-deficient.

Copper supplementation partially alleviated the growth depression due to 500 ppm zinc in the present study and there was no difference between the two levels of copper supplementation (Tables 4 and 5). Additionally, at 250 ppm zinc, growth was depressed only if dietary copper was marginally deficient. This protection by copper against excess zinc-caused growth depression has been reported previously (3, 14). On the other hand, Van Reen (2) and Cox and Harris (9) reported that copper had little influence on the growth-depressing action of 5000 and 4000 ppm zinc, respectively, and Campbell and Mills (15) found that supplementary copper prevented the development of zinc-induced copper deficiency in pregnant sheep, as measured by decreased plasma copper, but failed to prevent the adverse effect of high zinc (750 ppm) on weight gain. The increased body weight with iron supplementation, in the absence of excess zinc, has not been observed in other experiments.

Critical comparisons among studies are difficult, as levels of interacting trace elements in the diets used often were either not specified or were provided by a salt mix which has levels of most elements in excess of requirements. Therefore, pertinent interactions among the trace elements are difficult to evaluate. In this study the control diet contained zinc, iron, manganese, copper, and magnesium at requirement levels only. Thus, the birds fed this diet were more sensitive to any antagonism that resulted in an induced deficiency of any of these elements. The young quail also is sensitive to nutrient imbalances because of its rapid growth rate.

It is difficult to determine the implications of these animal data to humans. There is a lack of data on human body stores of copper. There are reports of marginally low intake of copper based on comparisons with the estimated safe and adequate daily intake range for copper (31). It is generally considered that a significant portion of children and of women of child bearing ages have insufficient stores of iron. The results of these animal studies support the need for further evaluation of the impact of zinc on human copper and iron status.

REFERENCES

1. Smith. S.E. and Larson, E.J. Zinc toxicity in rats. Antagonistic effects of copper and liver. J. Biol. Chem. 173: 29-38, 1946.

2. VanReen, R. Effects of excessive dietary zinc in the rat and the interrelationship with copper. Arch. Biochem. Biophys. 46: 337-344, 1953.

3. Grant-Frost, D.R. and Underwood, E.J. Zinc toxicity in the rat and its interrelation with copper. Austral. J. Exp. Biol. 36: 339-346, 1958.

4. Magee, A.C. and Matrone, G. Studies on growth, copper metabolism and iron metabolism of rats fed high levels of zinc. J. Nutr. 72: 233-242, 1960.

5. Settlemire, C.T. and Matrone, G. In vivo interference of zinc with ferritin iron in the rat. J. Nutr. 92: 153-158, 1967.

6. Settlemire, C.T. and Matrone, G. In vivo effect of zinc on iron turnover in rats and life span of the erythrocyte. J. Nutr. 92: 159-164, 1967.

7. Coleman, C.B. and Matrone, G. In vivo effect of zinc on iron-induced ferritin synthesis in rat liver. Biochim. Biophys. Acta 177: 106-112, 1969.

8. Cox, D.H. and Harris, D.L. Reduction of liver xanthine oxidase activity and iron storage proteins in rats fed excess zinc. J. Nutr. 78: 413-418, 1962.

9. Cox, D.H. and Harris, D.L. Effects of excess dietary zinc on iron and copper in the rat. J. Nutr. 70: 514-520, 1960.

10. Whanger, P.D. and Weswig, P.H. Effect of zinc on intracellular distribution of hepatic copper in rats. J. Nutr. 101: 1093-1098, 1971.

11. Cox, D.H., Schlicker, S.A. and Chu, R.C. Excess dietary zinc for the maternal rat, and zinc, iron, copper, calcium, and magnesium content and enzyme activity in maternal and fetal tissues. J. Nutr. 98: 459-466, 1969.

12. Chu, R.C. and Cox, D.H. Zinc, iron, copper, calcium, cytochrome oxidase and phospholipid in rats of lactating mothers fed excess zinc. Nutr. Rep. Int. 6: 61-66, 1972.

13. Campbell, J.K. and Mills, C.F. Effects of dietary cadmium and zinc on rats maintained on diets low in copper. Proc. Nutr. Soc. 33 (1): 15A-17A, 1974.

14. Hamilton, R.P., Fox, M.R.S., Fry, B.E. Jr., Jones, A.O.L. and Jacobs, R.M. Zinc interference with copper, iron and manganese in young Japanese quail. J. Food Sci. 44: 738-741, 1979.

15. Campbell, J.K. and Mills, C.F. The toxicity of zinc to pregnant sheep. Environ. Res. 20: 1-13, 1979.

16. Hill, C.H. and Matrone, G. A study of copper and zinc interrelationships. Proc. Twelfth World's Poultry Cong., pp. 219-222, 1962.

17. Hill, C.H. and Matrone, G. Chemical parameters in the study of in vivo and in vitro interactions of transition elements. Fed. Proc. 29: 1474-1481, 1970.

18. Porter, K.J., McMaster, D., Elmers, M.E. and Love, A.H.G. Anemia and low serum-copper during zinc therapy. Lancet 2: 774, 1977.

19. Petrie, J.J.B. and Row, P.G. Dialysis anaemia caused by subacute zinc toxicity. Lancet 1: 1178-1180, 1977.

20. Prasad, A.S., Brewer, G.J., Schoolmaker, E.B. and Rabbani, P. Hypocupremia induced by zinc therapy in adults. JAMA 240: 2166-2168, 1978.

21. Abdulla, M. Copper levels after oral zinc. Lancet 1: 616, 1979.

22. Rains, T.C., Zittel, H.E. and Ferguson, M. Elimination of anionic interferences in the flame spectrophotometric determination of calcium. Talanta 10: 367-374 ,1963.

23. Walpole, R.E. Introduction to Statistics, 2nd ed. Macmillan Publishing Co., Inc., New York, N.Y. 1974.

24. Bradley, J.V. Distribution-Free Statistical Tests. Prentice Hall, Inc., Englewood Cliffs, New Jersey, 1968.

25. Stewart, A.K. and Magee, A.C. Effect of zinc toxicity on calcium, phosphorus, and magnesium metabolism of young rats. J. Nutr. 82: 287-295, 1964.

26. Fox, M.R.S., Fry, B.E. Jr., Rothlein, J.E. and Hamilton, R.P. Magnesium bioassay in the Japanese quail. Fed. Proc. 35: 774, 1976.

27. Hamilton, R.P., Fox, M.R.S. and Fry, B.E. Jr. Unpublished data. Food and Drug Administration, Washington, D.C. 1976.

28. Osaki, S., Johnson, D.A. and Frieden, E. The possible significance of the ferrous oxidase activity of ceruloplasmin in normal human serum. J. Biol. Chem. 241: 2746-2751, 1966.

29. Evans, J.L. and Abraham, P.A. Anemia, iron storage and ceruloplasmin in copper nutrition in the growing rat. J. Nutr. 103: 196-201, 1973.

30. O'Dell, B.L. Biochemistry of copper. Med. Clinics N. America 60: 687-703, 1976.

31. National Research Council. Recommended Dietary Allowances, 9th ed. NAS, Washington, D.C. 1980.

ACKNOWLEDGMENTS

Some of the data were presented at the 41st Annual Meeting of the Institute of Food Technologists, Atlanta, GA, June 7-10, 1981. The authors thank Mildred Johnson, Candelia Fletcher, and James Etherith for their technical assistance.

NUTRITION RESEARCH, Vol. 1, pp. 601-607, 1981
0271-5317/81/060601-07$02.00/0 Printed in the USA.
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ROLE FOR FATTY ACID BINDING PROTEIN IN THE REGULATION OF HEPATIC LIPOGENESIS BY DIETARY LINOLEIC ACID

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ABSTRACT

The effect of added dietary ethyl palmitate or ethyl linoleate on hepatic lipogenic enzymes and the amount of hepatic fatty acid binding protein was examined in meal-fed rats. Dietary linoleate reduced the activities of fatty acid synthetase, glucose-6-phosphate dehydrogenase, NADP-malic enzyme, ATP-citrate lyase, and the amount of fatty acid binding protein compared to rats fed a fat-free or palmitate supplemented diet. The possible role of fatty acid binding protein in the regulation of lipogenesis by dietary fat is discussed.

Key Words - Lipogenesis; lipogenic enzymes, fatty acid binding protein, polyunsaturated fatty acids.

INTRODUCTION

The rate of hepatic fatty acid synthesis and the amounts of the lipogenic enzymes present are regulated by both the amount and type of dietary fat. It has generally been observed that when present in the diet at low levels, polyunsaturated fatty acids reduce lipogenesis *in vivo* and the activities of the lipogenic enzymes to a greater extent than do saturated fatty acids. The mechanism by which polyunsaturated fatty acids reduce hepatic lipogenesis remains unclear. Jeffcoat and James (1) have proposed that it is mediated by the activity of stearoyl CoA desaturase, the activity of which is reduced prior to that of fatty acid synthetase or acetyl CoA carboxylase in linoleate fed rats. The resulting increase in stearate could initiate the eventual change in the amounts of the lipogenic enzymes. A possible mediator of this effect is long-chain acyl CoA which should increase in these circumstances.

Long-chain CoA may be responsible for repression of acetyl CoA carboxylase synthesis and stimulation of its lysosomal degradation (2-8). However, several investigators have not found a relationship between long-chain CoA and lipogenesis (9-11). The cytosolic concentration of long-chain CoA can be altered by binding to fatty acid binding protein which also binds long chain acyl CoA. A role for this protein in the metabolism of fatty acids has been proposed (12-15).

The present experiments were designed to infestigate the hypothesis that in the liver of rats fed polyunsaturated fatty acids there is less binding of long-chain acyl-CoA to fatty acid binding protein. As a result, even though the total long-chain acyl CoA concentration is not changes, its effective concentration could be higher in rats fed polyunsaturated fatty acids.

MATERIALS AND METHODS

Male Sprague-Dawley rats weighing ∿200g were used in the study. Prior to the experimental period the rats had been fed lab chow. Six rats were assigned to each experimental diet. The rats were housed individually in plastic metabolic cages and maintained at a temperature of 25°C in a light controlled room (lights on 0800-200). The rats were fasted 48 hours and then fed the experimental diets (Table 1) for 5 days. All animals were studied simultaneously. The animals were fed 9.0g of food each morning at 0800 and they had consumed all the food by 1100. The rats were sacrificed between 1100 and 1200 on the fifth day. This feeding regimen resulted in equal carbohydrate intake by all three groups and equivalent absorbed doses of fatty acid in the palmitate- and linoleate-fed groups. Thus any differences observed can be attributed to the nature of the dietary fatty acids.

TABLE 1

Composition of Experimental Diets (g/100g)[1]

	Fat-Free	Palmitate	Linoleate
Casein	20	20	20
Glucose	60	60	60
AIN Mineral Mix[2]	3.5	3.5	3.5
AIN Vitamin Mix[2]	1.0	1.0	1.0
Choline Cl	0.2	0.2	0.2
DL-methionine	0.3	0.3	0.3
alpha cellulose	15	5	10
ethyl ester of fatty acid	--	10	5

The rats were sacrificed by decapitation. The liver was removed, rinsed and homogenized in 2 ml/g liver of 0.15M sucrose containing 10mM potassium phosphate, pH 7.4. The homogenate was centrifuged at 100,000 x g for 90 min at 4°C. A portion of the supernatant was used for determination of enzyme activities and for measurement of fatty acid binding protein. Fatty acid synthetase, glucose-6-phosphate dehydrogenase, malic enzyme, ATP-citrate lyase and glucokinase were determined as previously described (17). Fatty acid binding protein was determined as described by Mishkin et al. (18). Briefly, 4ml of supernatant was mixed with 3 nmoles of ^{14}C labelled palmitoyl CoA and incubated 30 min at 4°C. The sample was then applied to a Sephadex G-100 column (2.5 x 43 cm). The column was eluted with 0.1M NaCl containing 0.01M phosphate buffer pH 7.4. 6ml fractions were collected. A 1ml aliquot of each fraction was added to scintillation fluid and the radioactivity determined by liquid scintillation spectrometry. From this, the percentage of the added palmitoyl CoA which was bound by the fatty acid binding protein region (the peak eluting between fractions 23 and 35) was calculated. Data were statistically evaluated by analysis of variance. Treatment differences were ascertained using Duncans multiplerange test (24).

1. All diet components were purchased from ICN Pharmaceuticals, Cleveland, Ohio, except ethyl palmitate and linoleate which were obtained from Sigma Chemical Co.
2. Mixture 76™.

Table 2. Enzyme activities and fatty acid binding protein in the liver of rats fed diets containing no added fatty acid or added palmitate or linoleate.

	Fatty Acid Synthetase	Glucose-6-Phosphate Dehydrogenase	Malic Enzyme	ATP-Citrate Lyase	Glucokinase	Fatty Acid Binding Protein
Fat free	23.0 ± 7.8	133 ± 39	65.8 ± 23.9	50.0 ± 16.1	55.4 ± 5.7	37.1 ± 8.6
Palmitate	22.0 ± 7.4	175 ± 70	75.9 ± 30.1	55.0 ± 17.2	47.2 ± 4.2*	38.8 ± 4.6
Linoleate	12.2 ± 2.5*	73 ± 20*	35.5 ± 14.7*	32.0 ± 12.7*	40.4 ± 7.7*	27.3 ± 1.8*

Values are mean ± S.D. for 6 rats. Details of analysis are given in the Materials and Methods section. Enzyme activities are expressed as nmol substrate converted/mg protein/min. Fatty acid binding is expressed as the percentage of added palmityl-CoA bound. *$P < 0.05$ compared with fat-free group.

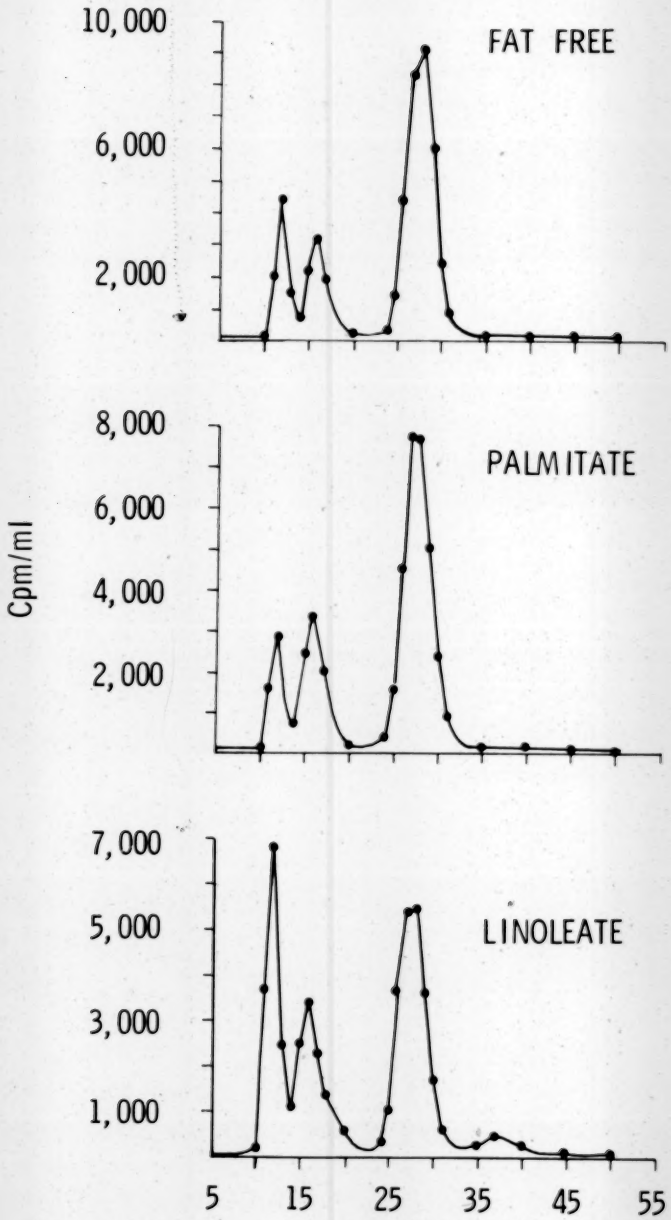

Figure 1. Binding of [^{14}C] palmityl CoA to the proteins in rat liver supernatant after chromatography on Sephadex G-100. The rats had been previously fed either a fat-free diet or fat-free diet containing ethyl palmitate or ethyl linoleate. Fatty acid binding protein was eluted in fractions 23-35.

RESULTS

The results are summarized in Table 2 and illustrated in Figure 1. In agreement with the results of Clarke et al. (11, 16, 19) when fed at low, equivalently absorbed levels, linoleate reduces the activities of fatty acid synthetase, glucose-6-phosphate dehydrogenase, malic enzyme and ATP-citrate lyase; but palmitate was without effect. In contrast to Clarke's results we observed a small but significant decrease in glucokinase when either fatty acid was included in the diet.

Typical elution patterns of liver fatty acid binding protein from rats fed each of the diets are shown in Figure 1. There is significantly less binding of palmityl CoA to fatty acid binding protein from rats fed linoleate compared to those fed palmitate or a fat-free diet. Iritani et al. (15) have shown that binding in this region correlates well with immunoprecipitable fatty acid binding protein, so we conclude that the livers of linoleate fed rats contain less fatty acid binding protein than those fed a fat-free or palmitate containing diet.

DISCUSSION

There is considerable evidence for a role for long-chain acyl CoA in the regulation of hepatic fatty acid synthesis. An inverse relationship between tissue long-chain CoA and fatty acid synthesis has been reported (3-7). In addition, there is good evidence for a specific role for long-chain CoA (at physiological concentrations) as an inhibitor of acetyl CoA carboxylase (20,21). Acetyl CoA carboxylase is generally accepted as the rate limiting step in fatty acid synthesis.

There is also experimental support for a role for long-chain CoA in the long term regulation of acetyl CoA carboxylase. Tanabe et al. (2) have observed an increased rate degradation of acetyl CoA carboxylase by lysosomal extracts in the presence of palmityl CoA and Kamiryo et al. (8) have found that long-chain acyl CoA is essential for repression of acetyl CoA carboxylase in *Candida lipolytica*. Fatty acyl CoA has also been observed to inactivate glucose-6-phosphate dehydrogenase *in vitro* and there is evidence for the presence of a long-chain acyl CoA inactivated form of glucose-6-phosphate dehydrogenase *in vivo* (22).

However, not all investigators have observed a relationship between long-chain CoA and fatty acid synthesis (9,10). In particular, with regard to inhibition of hepatic fatty acid synthesis by dietary linoleate, Clarke et al. (11) did not observe any difference in liver long-chain CoA in rats fed a fat-free diet or 3% linoleate, however, linoleate did reduce lipogenesis and the associated enzymes.

Fatty acid binding protein has been implicated as playing a regulatory role in lipogenesis, perhaps by mediating the effects of long-chain CoA (12,13). Lunzer et al. (14) found that fatty acid binding protein protects acetyl CoA carboxylase from inhibition by palmityl CoA and Iritani et al. (15) found more binding protein in rats fed a fat-free diet than in rats which had been fasted. The observations that fatty acid binding protein increases triglyceride synthesis and stimulates dialglycerol acyl transferase (15,23) suggests a role for fatty acid binding protein in the regulation of triglyceride synthesis.

Although there is no change in total long-chain CoA in linoleate fed rats (11), our finding of lower fatty acid binding protein in linoleate fed rats suggests that there would be a higher level of free long-chain CoA which could effectively inhibit and repress acetyl CoA carboxylase, glucose-6-phosphate dehydrogenase and perhaps other lipogenic enzymes.

We suggest the following sequence for the regulation of hepatic fatty acid synthesis by linoleate. As suggested by Jeffcoat and James (1), early inhibition of stearoyl CoA desaturase would cause a transient increase in saturated fatty acid from *de novo* synthesis which would also increase long-chain CoA and inhibit acetyl CoA carboxylase. We suggest that fatty acid binding protein is also

repressed. Continued linoleate feeding which would maintain normal total long-chain CoA levels could repress acetyl CoA carboxylase and possibly the other lipogenic enzymes due to the higher free long-chain CoA. Thus, long-chain CoA would play a role in regulation of fatty acid synthesis by dietary linoleate in both the short and long term.

Long chain fatty acids in the diet are transported as chylomicrons. The chylomicron fatty acids from the triglycerides are taken up primarily by adipose tissue. The chylomicron remnants are catabolized by the liver. Which source of fatty acids (triglycerides, phospholipids, cholesterol esters or depot fatty acids) is responsible for the repression of hepatic fatty acid synthesis and fatty acid binding protein will be the subject of future experiments.

ACKNOWLEDGEMENTS

The work described was supported by a grant from the Medical Research Council of Canada (MA-6097).

REFERENCES

1. JEFFCOAT, R. and JAMES, A.T. The control of stearoyl-CoA desaturase by dietary linoleic acid. FEBS Letters,85: 114-118, 1978.

2. TANABE, T., WADA, K., OGIWARA, H. and NUMA, S. Effects of allosteric effectors on proteolysis of rat liver acetyl CoA carboxylase by lysosomal extracts. FEBS Letters,82: 85-88, 1977.

3. BORTZ, W.M. and LYNEN, F. The inhibition of acetyl CoA carboxylase by long chain acyl CoA derivatives. Biochem. Z.,339: 77-82, 1963.

4. GUYNN, R.W., VELOSO, P. and VEECH, R.L. The concentration of malonyl-CoA and the control of fatty acid synthesis. J. Biol. Chem.,247: 7324-7331, 1972.

5. GOODRIDGE, A.G. Regulation of fatty acid synthesis in isolated hapatocytes prepared from livers of noenatal chicks. J. Biol. Chem.,248: 1924-1931, 1973.

6. GOODRIDGE, A.G. Regulation of fatty acid synthesis in isolated hapatocytes. Evidence for a physiological role for long chain fatty acyl coenzyme A and citrate. J. Biol. Chem.,248: 4319-4326, 1973.

7. NISHIKORI, K., IRITANI, N. and NUMA, S. Levels of acetyl coenzyme A carboxylase and its effectors after short term fat free refeeding. FEBS Letters, 32: 19-21, 1973.

8. KAMIRYO, T., NISHIKAWA, Y., MISHINA, M., TERAO, M. and NUMA, S. Involvement of long-chain acyl coenzyme A for lipid synthesis in repression of acetyl-coenzyme A carboxylase in Candida lipolytica. Proc. Natl. Acad. Sci. USA, 76: 4390-4394, 1979.

9. JACOBS, R.A. and MAJERUS, P.W. The regulation of fatty acid synthesis in human skin fibroblasts. J. Biol. Chem.,248: 8392-8401, 1974.

10. HALESTRAP, A.P. and DENTON, R.M. Hormonal regulation of adipose tissue acetyl coenzyme A carboxylase by changes in the polymeric state of the enzyme. Biochem. J.,142: 1277-1287, 1977.

11. CLARKE, S.D., ROMSOS, D.R. and LEVEILLE, G.A. Influence of dietary fatty acids on liver and adipose tissue lipogenesis and on liver metabolites in meals fed rats. J. Nutr.,107: 1277-1287, 1977.

12. OCKNER, R.K. and MANNING, J.A. Fatty acid binding protein. Role inesterification of absorbed long chain fatty acid in rat intestine. J. Clin. Invest.,58: 631-641, 1976.

13. OCKNER, R.K., MANNING, J.A., POPPENHAUSEN, R.B. and HO, W.K.C. A binding protein for fatty acids in cytosol of intestinal mucosa, liver, myocardium and other tissues. Science,177: 56-58, 1972.

14. LUNZER, M.A., MANNING, J.A. and OCKNER, R.K. Inhibition of rat liver acetyl coenzyme A and fatty acid. J. Biol. Chem.,252: 5483-5487, 1977.

15. IRITANI, N., FUKUDA, E. and INOGUCHI, K. A possible role of Z protein in dietary control of hepatic triacyl glycerol synthesis. J. Nutr. Sci. Vitaminol.,26: 271-277, 1980.

16. CLARKE, S.D., ROMSOS, D.R. and LEVIELLE, G.A. Differential effects of dietary methylesters of long chain saturated and polyunsaturated fatty acids on rat liver and adipose tissue lipogenesis. J. Nutr.,107: 1170-1181, 1977.

17. HERZBERG, G.R. and ROGERSON, M. Dietary corn oil does not suppress the fructose induced increase in hepatic fatty acid synthesis. Nutr. Res.,1: 73-82, 1981.

18. MISHKIN, S. and TURCOTTLE, R. The binding of long chain fatty acid CoA to Z, a cytoplasmic protein present in liver and other tissues of the rat. Biochem. Biophys. Res. Comm., 57: 918-926, 1977.

19. CLARKE, S.D., ROMSOS, D.R. and LEVEILLE, G.A. Time sequence of changes in hepatic fatty acid synthesis in rats meal fed polyunsaturated fatty acids. J. Nutr., 107: 1468-1476, 1977.

20. OGIWARA, H., TANABE, T., NIKAWA, J. and NUMA, S. Inhibition of rat liver acetyl-coenzyme A-carboxylase by palmitoyl coenzyme A. Formation of equimolar enzyme-inhibitor complex. Eur. J. Biochem.,89: 33-41, 1978.

21. NIKAWA, J., TANABE, T., OGIWARA, H., SHIBA, T. and NUMA, S. Inhibitory effects of long chain acyl coenzyme A analogues of rat liver acetyl coenzyme A carboxylase. FEBS Letters,102: 223-226, 1979.

22. DAO, M.W., WATSON, J.J., DELANEY, R. and JOHNSON, B.C. Purification of a new high activity form of glucose-6-phosphate dehydrogenase from rat liver and the effect of enzyme inactivation on its immunochemical reactivity. J. Biol. Chem.,254: 9441-9447, 1979.

23. O'DOHERTY, P.J.A. and KUKSIS, A. Stimulation of triacylglycerol synthesis by Z protein in rat liver and intestinal mucosa. FEBS Letters,60: 256-258, 1975.

24. STEEL, R.G.D. and TORRIE, J.H. Principles and Procedures of Statistics, McGraw Hill Book Company, New York, 1960.

NUTRITION RESEARCH, Vol. 1, pp. 609-616, 1981
0271-5317/81/060609-08$02.00/0 Printed in the USA.
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CELLULAR RESPONSES TO HIGH ASCORBIC ACID INTAKE BY NORMALLY NOURISHED AND UNDERNOURISHED MICE[1]

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ABSTRACT

Swiss albino mice were reared in litters of 8 or 14 pups each to produce normally nourished or undernourished pups, respectively. Litter size was maintained 26 days postnatally when several mice were sacrificed. The undernourished mice weighed less and had lower ascorbic acid contents in their livers and adrenals than normally nourished mice. They also had lighter brains, livers, and kidneys, and fewer cells (DNA) in those tissues. Animals remaining in both size litters were allowed free access to a stock diet and either untreated drinking water or drinking water containing 250 mg ascorbic acid per 100 ml for 28 days, when they were sacrificed. Supplementary ascorbic acid had no effect on cell number (DNA) or cell size (protein/DNA) in tissues of either normally nourished or early undernourished mice. Undernourished mice, however, retained fewer cells in their tissues than normally nourished mice.
KEY WORDS: ascorbic acid cellular growth early undernutrition

INTRODUCTION

Ascorbic acid seems to play a beneficial role in various forms of stress. Nandi and coworkers found that ascorbic acid reduced urinary histamine excretion from rats exposed to temperature stress, toxoid administration, or poor quality diet (1). Ascorbic acid reduced the growth retardation caused by mineral toxicities in the chick (2). Mice receiving ascorbic acid displayed more circulating interferon after injection with murine leukemia virus than did unsupplemented mice (3). Soliman and coworkers (4) found that ascorbic acid reduced liver damage caused by carbon tetrachloride in mice.

The purpose of our study was to determine the influence of high ascorbic acid intake on the stress of early undernutrition in mice. Previous studies demonstrated that animals undernourished at an early stage of development exhibit permanent reduction in growth potential (5), fewer brain cells (5,6), and learning disabilities (6,7). Such observations naturally led to an investigation of substances that could possibly rehabilitate the undernourished animals.

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[1]Supported in part by NIH Biomedical Research Grant RR07036, and the Kansas Agricultural Experiment Station, Contribution No. 82-76-j.

Our interest in ascorbic acid effects on early undernutrition was stimulated by Paul and Duttagupta's (8) finding that a large dose of vitamin C exerted a beneficial effect on the reproductive organs of the underfed rat, but a toxic effect on the ad libitum-fed rat. In addition, ascorbic acid and dehydroascorbic acid may play a role in cell division (9,10).

Our experiment was designed to investigate the effects of high ascorbic acid intake on growth of normally nourished and early undernourished mice. Our measurements of growth included body weight gain, and cell size and cell numbers in brains, livers, and kidneys.

MATERIALS AND METHODS

Animals and experimental design. All animals used were obtained from our breeding colony of random-bred Swiss albino mice. Virgin female mice weighing 30-35g were bred to males in a two-to-one ratio; after two weeks, obviously pregnant females were transferred to individual, solid-bottom cages. Room conditions were 22°C with 12 hours of light and 12 hours of darkness. A stock diet (Purina Laboratory Chow, Ralson Purina Co., St. Louis, Mo.) and deionized drinking water were allowed ad libitum throughout the experiment unless otherwise stated.

The majority of pups were born within a 48-hour interval approximately 3 weeks following matings. On the day of birth, litter size was adjusted to either 8 or 14 by redistributing pups born the same day. The pups in litters of 8 were considered normally nourished animals; those in litters of 14, undernourished. Pups were weighed twice weekly, and constant litter size was maintained throughout the lactation period.

When 26 days of age, two randomly selected male pups from each litter were fasted 12 to 14 hours and sacrificed by cervical dislocation. Their tissues were used for either ascorbic acid determinations; or for DNA, RNA, and protein determinations. At sacrifice brains, livers, and kidneys were removed and frozen in liquid nitrogen. Adrenals were immersed into 1.5 ml cold (4°C) 5% trichloracetic acid for immediate determination of ascorbic acid. Other tissues were stored at -18°C until analyzed.

The remaining males from both size litters were subdivided into two groups equalized for weight. Mice had free access to the stock diet and either untreated drinking water or drinking water containing 250 mg ascorbic acid per 100 ml water. The ascorbate solution had been used previously for mice (11,12), and we found that it did not restrict water intake. Experimental animals received the supplementary ascorbic acid fresh daily and consumed approximately 5 ml per day, equivalent to 12 mg in the supplemented groups.

After 28 days the mice were sacrificed and tissues were obtained as for the 26-day sacrifice. DNA, RNA, and protein determinations were performed to see if high ascorbic acid intake influenced cellular growth in the normally nourished or undernourished animals.

Biochemical analyses. Ascorbic acid contents of tissues were determined by the 2,4 dinitrophenylhydrazine method of Bessey (13) on the day of sacrifice.

Tissues used for nucleic acid and protein determinations were homogenized in cold deionized water. The fraction containing the nucleic

acids and proteins was precipitated with 10% trichloroacetic acid and solutions were placed in a 95°C water bath for 15 minutes to hydrolyze the precipitate. The soluble fraction was assayed for DNA by the diphenylamine reaction (14), and RNA by the orcinol reaction (15). The contribution of DNA to the orcinol color reaction was also determined.

Because they were more easily solubilized and less expensive than the nucleic acids, standards used were deoxyribose after calibration against highly polymerized calf thymus DNA; and ribose after calibrating against calf liver RNA. Nucleic acids were obtained from Sigma Chemical Co., St. Louis, Mo. Protein contents were determined in tissue homogenates by the biuret method (16) using bovine serum albumin as the standard.

Statistical analyses. Ascorbic acid and litter size effects were determined by Least Significant Differences Tests following significant ($P<0.05$) Analysis of Variance procedures (17).

RESULTS

Several differences between the normally nourished (8/litter) and the undernourished mice (14/litter) were apparent at weaning (26 days of age). The undernourished mice had a lower concentration of liver ascorbic acid and total adrenal ascorbic acid than the normally nourished mice (Table 1). Early undernutrition appeared not to have influenced ascorbic acid concentrations in the brain or kidneys.

TABLE 1

Ascorbic acid content in tissues of normally nourished and undernourished mice at weaning[1]

Tissue	8/litter	14/litter
Liver (mg/100g tissue)	35.41	29.24[a]
Kidney (mg/100g tissue)	21.96	26.47
Brain (mg/100g tissue)	55.58	52.62
Adrenal (µg total)	12.86	8.57[a]

[1]Mean for 6 mice at 26 days of age. [a]$P<0.05$

Undernourished mice also weighed less at weaning, approximately 47% of those normally nourished (Table 2). Their mean liver weights were 38%, kidneys 47%, and brains 83% of the weights of normally nourished mice organs. We performed DNA, RNA, and protein determinations on brains, livers, and kidneys so we could determine cell number and cell size in those tissues. At weaning the undernourished mice had significantly less total DNA in their brains, livers, and kidneys than the normally nourished animals, indicating that undernutrition reduced cell number in these tissues. Our findings are similar to those reported by Winick for rats (5). We also found lower protein/DNA ratios in livers and kidneys of undernourished mice than in those of normally nourished mice, indicating that these cells were smaller in undernourished animals at weaning.

TABLE 2

Body weight and tissue composition in normally nourished and undernourished mice at weaning[1]

Measurement	8/litter	14/litter
Fasted body wt. (g)	12.7	6.0[a]
	Brain	
Brain wt. (g)	0.42	0.35[a]
Brain, % of body wt.	3.34	6.16[a]
DNA, mg/g tissue	3.04	3.33
DNA, mg/brain	1.29	1.13[a]
RNA, mg/g tissue	8.23	8.60
Protein, mg/g tissue	126.4	127.7
Protein/DNA	42.1	39.3
	Liver	
Liver wt. (g)	0.61	0.23[a]
Liver, % of body wt.	4.78	3.72[a]
DNA, mg/g tissue	5.76	7.69[a]
DNA, mg/liver	3.77	1.48[a]
RNA, mg/g tissue	27.17	20.23[a]
Protein, mg/g tissue	279.9	252.5
Protein/DNA	49.8	33.4[a]
	Kidney	
Kidney wt. (g)	0.17	0.08[a]
Kidney, % of body wt.	1.35	1.38
DNA, mg/g tissue	9.24	11.81[a]
DNA, mg/kidneys	1.65	0.90[a]
RNA, mg/g tissue	12.88	12.66
Protein, mg/g tissue	255.3	198.7[a]
Protein/DNA	27.8	17.2[a]

[1]Mean for 7-8 mice at 26 days of age. [a]$P<0.05$

The remaining mice from both size litters were given stock diet and either untreated drinking water or the ascorbate solution for the next 28 days, when they were sacrificed (54 days of age). Ascorbic acid and litter size effects are shown in Table 3. Comparing unsupplemented and supplemented animals within each litter size group showed no significant ascorbic acid effects, but body weights, weights of brains, livers and kidneys, and total DNA contents were generally lower in undernourished mice than in normally nourished mice at 54 days of age, regardless of ascorbic acid treatment.

TABLE 3

Ascorbic acid (AsA) and litter size influences on body weight and tissue composition[1]

Measurement	8/litter		14/litter	
	-AsA	+AsA	-AsA	+AsA
Fasted body wt. (g)	29.4	27.8	23.9[a]	24.3[a]
		Brain		
Brain wt. (g)	0.48	0.48	0.43[a]	0.43[a]
Brain, % of body wt.	1.64	1.71	1.80	1.76
DNA, mg/g tissue	3.27	3.08	3.17	3.14
DNA, mg/brain	1.52	1.44	1.35[a]	1.32[a]
RNA, mg/g tissue	8.31	8.84	8.88	8.63
Protein, mg/g tissue	145.7	152.6	162.6	156.5
Protein/DNA	45.6	50.0	51.6	57.5
		Liver		
Liver wt. (g)	1.48	1.42	1.28[a]	1.31
Liver, % of body wt.	5.05	5.12	5.30	5.39
DNA, mg/g tissue	5.14	5.28	4.86	5.13
DNA, mg/liver	7.63	7.52	6.22[a]	6.49[a]
RNA, mg/g tissue	24.10	24.78	24.61	26.53
Protein, mg/g tissue	384.1	373.8	316.2[a]	323.0[a]
Protein/DNA	76.4	74.2	71.0	65.2
		Kidney		
Kidney wt. (g)	0.48	0.49	0.36	0.40
Kidneys, % of body wt.	1.63	1.74	1.54	1.67
DNA, mg/g tissue	6.35	6.00	6.90	6.00
DNA, mg/kidneys	3.01	2.90	2.50[a]	2.40[a]
RNA, mg/g tissue	14.08	13.48	13.80	13.87
Protein, mg/g tissue	354.1	348.0	324.9	318.7
Protein/DNA	56.7	54.7	49.2	55.2

[1]Ascorbic acid administered for 28 days after early normal nutrition (8/litter) or undernutrition (14/litter). Values are mean for 7-8 mice. [a]$P<0.05$ when compared with normally nourished animals receiving the same ascorbic acid treatment. No significant ascorbic acid effects were observed among animals within each litter size group

DISCUSSION

It is clear that early undernutrition has many effects. We, like previous investigators (5,6) have shown that early undernutrition restricts whole body and cellular growth. Our data also indicate that early undernutrition reduces liver and adrenal ascorbic acid contents. Other researchers have found low ascorbic acid contents in livers and kidneys of rats fed a protein-restricted diet (18) and vitamin A-deficient diet (19). So dietary stress appears to reduce ascorbic acid contents in those tissues.

Although cellular growth has been well studied in rats (5), it is less clearly defined in mice. Existing data center largely on the mouse brain, which attains maximum cells (DNA) at about 18 days postnatally, only 1 or 2 days earlier than the rat brain (20). Information on cellular growth of livers and kidneys in mice is scarce, although one might expect development of mice and rats to parallel one another until weaning, as both are rodents with similar gestation and weaning periods.

Our study shows that growth of livers and kidneys in mice from 26 to 54 days old is characterized by an increase in cell numbers (hyperplasia) and in cell size (hypertrophy), but we noted little increase in brain cell numbers during this time interval, indicating that most of the brain cells in our animals were attained before day 26.

Our study reinforces previous findings that early undernutrition permanently decreases cell numbers in brain, liver, and kidneys (5). The undernourished animals, even after 28 days' free access to an adequate diet, maintained fewer cells in those tissues than did normally nourished mice.

Ascorbic acid administered postnatally from 26 to 54 days appeared not to influence either cell number or cell size in any tissues studied. Neither normally nourished animals nor undernourished mice were influenced. In a previous study (21) we found that 21-day-old weanling male mice fed this ascorbic acid regimen for 3 weeks had smaller body weights and livers than nonsupplemented mice when both groups were 6 weeks old. In the current study the normally nourished mice receiving the ascorbic acid had smaller bodies and livers but not significantly smaller. A possible explanation is that our mice were weaned 5 days later than in the previous study, and some growth occurred without ascorbic acid exposure. Normally we wean mice at 21 days but we felt that the mice from the larger litters would not survive independently of the mothers then, so we weaned them later.

We suspected that the undernourished mice would be influenced by high ascorbic acid intake because large doses of ascorbic acid have been shown to be beneficial in various dietary stresses in rats (1). On the other hand, one might also speculate that undernourished mice might be adversely affected by high ascorbic acid intake, because of probable deficiencies in metabolism and excretion. But we noted neither beneficial nor toxic effects of ascorbic acid on measurements in our undernourished mice.

It is possible that ascorbic acid, if administered concurrently with the period of undernutrition, might have different effects on cellular growth. One would then have to devise a way to supplement the pups during the preweaning period. Mice that age would be difficult to force-feed. Ascorbic acid might be given to the lactating mothers. We previously found (22), however, that this ascorbic acid mixture restricted the water intake of lactating female mice even though it did not influence intakes of

pregnant mice or growing male mice. So an alternate method of supplementation should be used. We hope to continue studies on ascorbic acid influences during various stages of growth.

ACKNOWLEDGMENT

The assistance of Dr. Arthur D. Dayton, Department of Statistics, in performing statistical analyses is gratefully acknowledged.

REFERENCES

1. NANDI, B.K., SUBRAMANIAN, N., MAJUMDER, A.K. and CHATTERJEE, I.B. Effect of ascorbic acid on detoxification of histamine under stress conditions. Biochem. Pharmacol. 23: 643-647, 1974.

2. HILL, C.H. Studies on the ameliorating effect of ascorbic acid on mineral toxicities in the chick. J. Nutr. 109: 84-90, 1979.

3. SIEGEL, B.V. Enhanced interferon response to murine leukemia virus by ascorbic acid. Infec. Immun. 10: 409-410, 1974.

4. SOLIMAN, M.A., DE HONDT, H.A. and ELWI, A.M. Vitamin E, nicotinic acid, vitamin C and total belladonna alkaloids as liver cell regenerators. Pharmacology. 2: 352-360, 1969.

5. WINICK, M. and NOBLE, A. Cellular response in rats during malnutrition at various ages. J. Nutr. 89: 300-306, 1966.

6. HOWARD, E and GRANOFF, D.M. Effect of neonatal food restriction in mice on brain growth, DNA and cholesterol, and on adult delayed response learning. J. Nutr. 95: 111-121, 1968.

7. LEATHWOOD, P., BUSH, M., BERENT, C. and MAURON, J. Effects of early malnutrition on Swiss white mice: Avoidance learning after rearing in large litters. Life Sci. 14: 157-162, 1974.

8. PAUL, P.K. and DUTTAGUPTA, P.N. Beneficial or harmful effects of a large dose of vitamin C on the reproductive organs of the male rat depending upon the level of food intake. Ind. J. Exp. Biol. 16: 18-21, 1978.

9. EDGAR, J.A. Is dehydroascorbic acid an inhibitor in the regulation of cell division in plants and animals. Experientia. 25: 1214-1215, 1969.

10. EDGAR, J.A. Dehydroascorbic acid and cell division. Nature. 227: 24-26. 1972.

11. FRITH, C.H., RULE, J. and KODELL, R.L. The effects of ascorbic acid on the induction of urothelial lesions in mice by 2-acetylaminofluorene. Toxicology Letters. 6: 309-318, 1980.

12. SCHLEGEL, J.U., PIPKIN, G.E., NISHIMURA, R. and SHULTZ, G.N. The role of ascorbic acid in the prevention of bladder tumor formation. Trans. Am. Assoc. Genitourin. Surg. 85-89, 1969.

13. BESSEY, O.A., LOWRY, O.H. and BROCK, M.J. The quantitative determination of ascorbic acid in small amounts of white blood cells and platelets. J. Biol. Chem. 168: 197-205, 1947.

14. PLUMMER, D.T. An Introduction to Practical Biochemistry, p. 215, McGraw-Hill Co.: New York, N.Y., 1971.

15. KERR, S.E. and SERAIDARIAN, K. The separation of purine nucleosides from free purines and the determination of the purines and ribose in these fractions. J. Biol. Chem. 159: 211-225, 1945.

16. PLUMMER, D.T. An Introduction to Practical Biochemistry, p. 156, McGraw-Hill Co.: New York, N.Y., 1971.

17. SNEDECOR, G.W. and COCHRAN, W.G. Statistical Methods, Iowa State University Press, Ames, IA, 1976.

18. MUKHERJEE, D., KAR, N.C., SASMAL, N. and CHATTERJEE, G.C. The influence of dietary protein on ascorbic acid metabolism in rats. Biochem. J. 106: 627-632, 1968.

19. MAYER, J. and KREHL, W.A. The relation of diet composition and vitamin C to vitamin A deficiency. J. Nutr. 35: 523-537, 1948.

20. WINICK, M. Nutrition and nerve cells. Fed. Proc. 29: 1510-1515, 1970.

21. GRUNEWALD, K.K. and MITCHELL, L.K. Large doses of vitamin C: Effect on growth, tissue concentration, and liver composition in mice. In review, 1981.

22. MITCHELL, L.K. and GRUNEWALD, K.K. Unpublished results, 1981.

NUTRITION RESEARCH, Vol. 1, pp. 617-622, 1981
0271-5317/81/060617-06$02.00/0 Printed in the USA.
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REVIEW ARTICLE

CHELATING AGENTS AND BIOAVAILABILITY OF MINERALS

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The effects of chelating agents on the absorption of mineral nutrients are reviewed. The evidence indicates that mineral chelates are not always better sources of minerals than the simple salts. The effect of chelating agents generally depends on the stability of the chelates in the intestine and their solubility in water or lipids. The stability of the chelates of heavy metals can be increased by excesses of chelating agents and the beneficial effect on absorption can be reversed. Many chelates of iron, zinc and copper are equally well utilized as the corresponding salts. Fe(III)-EDTA, Glucose Tolerance Factor (a chromium complex of nicotonic acid and aminoacids, which occurs in brewer's yeast) and a zinc chelate in human milk are better utilized by man than ferrous sulphate, chromium chloride or zinc sulphate respectively. Copper-NTA is a better source of copper than copper sulphate for crinkled mutant mice. Zinc absorption is inhibited by EDTA in man, but in animals EDTA increases bioavailability of phytate bound zinc. Although ascorbic acid promotes absorption of non-heme iron in man, it depresses the absorption of copper and it has no effect on that of zinc.

KEY WORDS: Mineral Chelates, Minerals, Chelating Agents, Bioavailability

In recent years, mineral chelates have been promoted as nutritional supplements. It is claimed, in the lay press, that the bioavailability of chelated mineral nutrients is much better than that of salts. For example, zinc methionate is claimed to be a better source of zinc than zinc sulphate. The evidence on the bioavailability of chelated mineral nutrients and the effect of chelating agents on the absorption of mineral elements is reviewed here.

A chelate is a weakly dissociated complex in which the metal is part of a ring structure. By extention this term is applied to chemical compounds in which a metallic ion is sequestered and bound into a ring within the chelating molecule, for example, ferrous fumerate or calcium gluconate. Iron chelates are useful in the treatment of iron deficiency anemia in cases where an iron salt has adverse gastrointestinal effects. Except for iron complexes, mineral chelates have received little attention by researchers in respect of their bioavailability in man. More work, however, has been reported on the absorption of chelated minerals in animals.

Chelating agents can be divided into two groups. 1. Those which form stable chelates in competition with cellular acceptor sites on the mucosa or other tissues. Such agents inhibit absorption of heavy metals. 2. Those which form weaker chelates, which keep the heavy metals in solution and release them tu the acceptor sites on the mucosa (1). Chelating agents of the first group are useful in the removal of heavy metals from the body in cases of toxicity whereas the group 2 agents, when added to diets, promote

absorption of heavy metals. It is, however, possible to increase the stability of a weak chelate by polymerization induced by excessive amounts of the chelating agent. Consequently, a promotor of absorption can become an inhibitor (1).

There are examples in the bacterial world where chelating agents such as enterochelin are secreted into the environment in order to bind iron which the bacteria require. In higher plants, the excretion of malonic or citric acid by the roots serves a similar purpose (2). In animals also, chelation plays an important role. For example cobalt can be utilized by simple stomached animals only when it is complexed in the corrin ring, which they are unable to synthesize. Microorganisms, however, have the capacity to synthesize the corrin ring. On the other hand, animals can synthesize the porphyrin ring which can chelate iron and form the heme moiety of the cytochromes (3).

IRON

Retention of iron from 14 radio-labelled compounds by men and women was determined by Brise and Hallberg (4), using ferrous sulphate as a standard source. The ferrous compounds tested viz. succinate, lactate, fumarate, glycine-sulphate, glutamate and glyconate, were as good a source of iron as ferrous sulphate. Iron from ferric compounds viz. cholineisocitrate, citrate and versenate was 25 to 40% as well absorbed as that from the standard source. In guinea pigs, however, retention of iron from ferric citrate, NTA (nitrilotriacetic acid), EDTA (ethylene diaminetetraacetic acid) and gluconate was equal to that of iron from ferrous sulphate, whereas ferric fructose resulted in a 50 to 100% better retention of iron (5). In this study Bates et. al. used ^{59}Fe labelled sources and whole body counting for the determination of iron retention. A similar effect of fructose on iron absorption by human subjects was reported earlier (6).

Layrisse and Martinez-Torres (7) showed that absorption of iron from Fe(III)-EDTA by Venezuelan men and women was about twice as high as that from ferrous sulphate ingested with typical local meals. The superiority of NaFeEDTA over $Fe_2(SO_4)_3$ as a source of iron for man was also reported by Viteri et al. (32). In a subseuqent paper Martinez-Torres et al. (8) reported that the absorption of iron from ferrous sulphate added to six different food vehicles, varied from 2 to 30%, whereas, the efficacy of Fe(III)-EDTA as a fortification iron source added to the same foods remained practically unchanged (8-13%). Nevertheless, Christopher et al. (2) had reported earlier that Fe(III)-EDTA was rapidly absorbed and excreted in urine by rats. In humans also, Cook and Monsen (9) observed a decrease in iron retention when EDTA was added to a standard or a semi-synthetic meal to give EDTA:Fe ratio of 1:1 and on increasing the ratio, retention decreased further. Similar results were reported by Forth et al (1) and Monsen and Page (33) in ligated intestinal segments of rats. The former group also observed that nicotinehydroxamic acid also exerted an inhibitory effect similar to EDTA. The discrepancy between the observation of Layrisse and Martinez-Torres (7) and that of Cook and Monsen (9) may be attributed to the fact that the former group used preformed Fe(III)-EDTA whereas the latter added EDTA to the diets.

Although ferric citrate was not found to be as good an iron source as ferrous sulphate for man (4), the absorption of iron from monoferrous citrate by Indian men and women was found to be equal to that from ferrous sulphate (10).

Iron proteinate (a protein hydrolysate containing 10% Fe in chelated form) added to the late gestation and lactation diet of sows at a level of 250 to 500 ppm Fe was not effective in preventing piglet anemia. The level of iron required for this purpose was very high (3000 ppm), probably indicating poor retention of iron from this source by the sows (11).

The effect of cysteine on the absorption of non-heme iron from a Venezuelan diet was investigated by Martinez-Torres et al. (12). They observed that addition of 210 or 630 mg of cysteine to a rural Venezuelan diet of black beans, maize and rice increased the absorption of non-heme iron by about 100%. Cysteine had a similar effect on the absorption of ferric iron. If the meal was cooked after adding cysteine, however, the beneficial effect on iron absorption was abolished, probably because of the oxidation of cysteine to cystine. Iron absorption was also increased by the addition of alanine, phenylalanine, histidine or 8-hydroxyquinoline to intestinal loops of rats but the sulphonic acid derivative of 8-hydroxyquinoline had an inhibitory effect.An excess of histidine also

had an adverse effect on iron absorption (2).

The promotion of iron absorption by ascorbic acid (33) was recently confirmed by Derman et al (13). The absorption of iron in Indian women given infant formula fortified with ferrous sulphate or infant cereals fortified with ferric ammonium citrate, ferrous sulphate or ferric pyrophosphate was less than 3.2%. When ascorbic acid was added at a molar ratio of iron to ascorbic acid of 1:1.5 iron absorption was increased 3-fold and a ratio of 1:3 caused the iron absorption to be greater than 6-fold.

ZINC

In chicks the bioavailability of zinc from $ZnCO_3$, ZnO, $ZnCl_2$ was equal to that of zinc from proteinates (14). Similarly, there was no difference in the increase in serum zinc of healthy human volunteers after they ingested either the N-acetylhydroxyproline salt of zinc or zinc sulphate (15). At a zinc to histidine molar ratio of 1:250 in intestinal loops, Schwarz and Kirchgessner (16) did not find any improvement in zinc absorption by rats. Without histidine, the absorption of zinc was higher from the duodenum than from the jejunum. When the zinc to histidine ratio was increased to 1:2000, absorption from the jejunum became equal to that from the duodenum.

An extensive investigation of the effect of zinc-ligand mixtures on serum zinc levels in rats was undertaken by Giroux and Prakash (17). They observed that lysine, cysteine and a mixture of glycine and histidine produced higher serum zinc than zinc sulphate. Phytic acid, aminopolycarboxylic acids (including disodium EDTA) and penicillamine signficantly suppressed increases in serum zinc concentration. Zinc absorption was increased when alanine, phenylalanine, histidine or 8-hydroxyquinoline was added to ligated intestinal segments of rats, but it was decreased by an excess of histidine or sulphonic acid derivative of 8-hydroxyquinoline or nicotinehydroxamic acid (1). Oral treatment of calves, suffering from zinc malabsorption, with 8-hydroxyquinoline resulted in a considerable increase in zinc absorption (18). The promotion of zinc absorption by cysteine was also observed in chicks (19) and the inhibitory effect of EDTA on zinc absorption was confirmed by Solomons et al. (20) in healthy men and women from Guatemala. In contrast to the effect observed with iron, absorption of zinc in man was not promoted by ascorbic acid (21). The therapeutic value of human milk in the treatment of acrodermatitis enteropathica was attributed by Lönnerdal et al. (22) to a higher level of a zinc binding ligand viz. citrate, in human milk than in cow's milk. Evans and Johnson (23), however, claim that the zinc binding ligand concerned is picolinic acid.

COPPER

Copper in the form of its oxide, hydroxide, iodide, glutamate, glycerophosphate, aspartate, citrate and pyrophosphate was well utilized by anemic rats for hemoglobin synthesis, whereas copper from copper sulphide and copper porphyrin was not available for this purpose (24). Similarly, there was no difference in the absorption of copper by rats from the citrate, fumarate, oxalate, EDTA or sulphate. Moreover, L-aminoacids facilitate copper absorption and with polymerization of the aminoacid the effect decreases (25). Adding alanine, phenylalanine, histidine or 8-hydroxyquinoline to ligated intestinal segment of rat intestine increased the absorption of copper. An excess of histidine, sulphonic acid derivative of 8-hydroxyquinolie or nicotinehydroxamic acid, however, caused a decrease in copper absorption (1). Whereas ascorbic acid promotes iron absorption and has no effect on zinc absorption in man, it decreases absorption and retention of copper in the chick, rabbit, pig and rat (26). Copper nitrilotriacetate was found to be a better source of copper than copper sulphate for crinkled mutant mice, which suffer from a genetic disorder of copper metabolism (27).

CHROMIUM

The availability of chromium in brewer's yeast is higher than that in many organic or inorganic compounds studied in animals and man. The chromium complex in brewer's

yeast has been named "Glucose Tolerance Factor (GTF)" because it has been shown to potentiate the action of insulin. GTF is a coordination compound consisting of chromium (III), nicotinic acid, glycine, glutamic acid and probably cysteine (28). Complexation with oxalate caused a significant increase in the absorption of Cr (III) by the rat but phytate decreased absorption. Citrate and EDTA, however, did not have any effect (34).

COBALT AND MANGANESE

The addition of alanine, phenylalanine, histidine or 8-hydroxyquinoline to ligated segments of rat intestine increased the absorption of cobalt but not manganese. On the other hand, the sulphonate of 8-hydroxyquinoline or an excess of the aminoacids inhibited cobalt absorption but had no effect on manganese absorption. EDTA and nicotine-hydroxamic acid decreased absorption of both cobalt and manganese. Moreover, with an increasing concentration of these agents the inhibition of absorption increased. The differences in the effects of different chelating agents on different minerals may be related to the stability of the chelates at the intestinal pH or their solubility in water or lipids (1).

CALCIUM AND MAGNESIUM

Absorption of calcium by rats was promoted by several aminoacids, lysine and arginine being the most effective. There was no difference between the abilities of D- and L-lysine to increase calcium absorption. The promotion of calcium absorption did not depend on the solubility of the compounds in water since calcium-glycine was very soluble but it was not particularly effective (29).

The effect of chelating agents on magnesium absorption has not received much attention. However, several magnesium salts including carbonate, chloride, oxide, phosphate, sulphate and silicate were found to be nearly equal in their ability to support growth and maintain plasma magnesium levels and the kidney magnesium concentration in weanling rats (30). The hydrochloride salt of magnesium aspartate was reported to be more effecive than the aspartate in the treatment of experimental cardiomyopathy in animals (31).

CONCLUSION

The effect of chelating agents on mineral absorption varies from one mineral to another and there are also differences between species. The concentration of a chelating agent in relation to that of a mineral nutrient or its state of polymerization determine whether promotion or inhibition of absorption will result. The absorption of a mineral from a preformed chelate will depend on its stability during its passage through the gastrointestinal tract.

REFERENCES

1. FORTH, W., NELL, G. and RUMMEL, W. Chelating agents and the transfer of heavy metals across the mucosal epithelium. In: Proceedings of University of Missouri's 7th Annual Conference on Trace Substances in Environmental Health. D.D. Hemphill (ed). Curators of the University of Missouri, Columbia, Missouri, 1973, pp. 339-345.

2. CHRISTOPHER, J.P., HEGENAUER, J.C. and SALTMAN, P.D. Iron metabolism as a function of chelation. In: Proceedings of the Second International Symposium on Trace Element Metabolism in Animals. W.G. Hoekstra, J.W. Suttie, H.E. Ganther and W. Mertz (eds). University Park Press, Baltimore, 1974, pp. 133-145.

3. O'DELL, B.L. Biological availability of trace elements as affected by chemical form. In: Proceedings of University of Missouri's 4th Annual Conference on Trace Substances in Environmental Health. D.D. Hemphill (ed). University of Missouri, Columbia, Missouri, 1971, pp. 209-210.

4. BRISE, H . and HALLBERG, L. Absorbability of different iron compounds. Acta Med. Scand. 171 (Suppl. 376) :23-37, 1962.

5. BATES, G.W., BOYER, J., HEGENAUER, J.C. and SALTMAN, P. Facilitation of iron absorption by ferric fructose. Am. J. Clin. Nutr. 25:983-986, 1972.

6. BRODAN, V., BRODANOVA, M., KUHN, E., KORDAC, V. and VALEK, J. Influence of fructose on iron absorption from the digestive system of healthy subjects. Nutr. Dieta 9:263-270, 1967,

7. LAYRISSE, M. and MARTINEZ-TORRES, C. Fe(III)-EDTA complex as iron fortification. Am. J. Clin. Nutr. 30:1166-1174, 1977.

8. MARTINEZ-TORRES, C., ROMANO, E.L., RENZI, M. and LAYRISSE, M. Fe(III)-EDTA complex as iron fortification. Further studies. Am. J. Clin. Nutr. 32:809-816, 1979.

9. COOK, J.D. and MONSEN, E.R. Food iron absorption in man II. The effect of EDTA on absorption of dietary non-heme iron. Am. J. Clin. Nutr. 29:614-620, 1976.

10. RAO, B.S.N., KATHOKE, S. and APTE, S.V. Mono ferrous citrate ($FeC_6O_7H_2O$) as an iron fortificant. Br. J. Nutr. 39:663-665, 1978.

11. MILLER, E.R., BRADY, P.S. and KU, P.K. Evaluation of iron proteinate for indirect prevention of baby pig anemia. In: Proceedings for the Third International Symposium on Trace Element Metabolism in Man and Animals. M. Kirchgessner (ed). Arbeitskreis für Tierernährungsforschung e.v. (ATW), Freising-Weihenstephan, 1978, pp. 506-510.

12. MARTINEZ-TORRES, C., ROMANO, E. and LAYRISSE, M. Effect of cysteine on iron absorption in man. Am. J. Clin. Nutr. 34:322-327, 1981.

13. DERMAN, D.P., BOTHWELL, T.H., MacPHAIL, A.P., TORRENCE, J.D., BEZWODA, W.R. CHARLTON, R.W. and MAYET, F.G.H. Importance of ascorbic acid in the absorption of iron from infant foods. Scand. J. Haematol. 25:193-201, 1980.

14. AMMERMAN, C.B. and MILLER, S.M. Biological availability of minor mineral ions: A review. J. Anim. Sci. 35:681-694, 1972.

15. SCHELLING, J.L., MULLER-HESS, S. and THONNEY, F. Effect of food on zinc absorption. Lancet. i:968-969, 1973.

16. SCHWARZ, F.J. and KIRCHGESSNER, M. Tierexperimentelle untersuchungen zur Zn-absorption bei verschiedenen dünndarmabschnitten und Zn-verbindungen. Nutr. Metab. 18:157-166, 1975.

17. GIROUX, E. and PRAKASH, N.J. Influence of zinc-ligand mixtures on serum zinc levels in rats. J. Pharmaceutcal Sci. 66:391-395, 1977.

18. FLAGSTAD, T. The influence of oxychinolines on intestinal zinc absorption and on zinc transport in serum in cattle. In: Proceedings of the Third International Symposium on Trace Element Metabolism in Man and Animals. M. Kirchgessner (ed). Arbeitskreis für Tierernährungsforschung Weihenstephan, Freising-Weihenstephan, 1978, pp. 423-425.

19. HOEKSTRA, W.G. The complexity of dietary factors affecting zinc nutrition and metabolism in chicks and swine. In: Proceedings of WAAP/IBP International Symposium on Trace Element Metabolism in Animals. C.F. Mills (ed). E. & S. Livingstone, Edinburgh, 1970, pp. 347-353.

20. SOLOMONS, N.W., JACOB, R.A., PINEDA, O. and VITERI, F.E. Studies on the bioavailability of zinc in man. Effects of the Guatemelan rural diet and of the iron-fortifying agent, NaFeEDTA. J. Nutr. 109:1519-1528, 1979.

21. SOLOMONS, N.W., JACOB, R.A. PINEDA, O. and VITERI, F.E. Studies on the bioavailability of zinc in man. III. Effects of ascorbic acid on zinc absorption. Am. J. Clin. Nutr. 32:2495-2499, 1979.

22. LÖNNERDAL, B., STANISLOWSKI, A.G. and HURLEY, L.S. Isolation of a low molecular weight zinc binding ligand from human milk. J. Inorg. Biochem. 12:71-78, 1980.

23. EVANS, G.W. and JOHNSON, E.C. Effect of iron, vitamin B-6 and picolinic acid on zinc absorption in the rat. J. Nutr. 111:68-75, 1981.

24. UNDERWOOD, E.J. Trace Elements in Human and Animal Nutrition. Academic Press, New York, 1977, p. 70.

25. KIRCHGESSNER, M. and GRASSMANN, E. The dynamics of copper absorption. In: Proceedings of WAAP/IBP International Symposium on Trace Element Metabolism in Animals. C.F. Mills (ed). E. & S. Livingstone, Edinburgh, 1970, pp. 277-287.

26. MASON, K.E. A conspectus of research on copper metabolism and requirement of man. J. Nutr. 109:1979-2066, 1979.

27. KEEN, C.L., SALTMAN, P. and HURLEY, L.S. Copper nitrilotriacetic acid: a potent therapeutic agent in the treatment of a genetic disorder of copper metabolism. Am. J. Clin. Nutr. 33:1789-1800, 1980.

28. KIENLE, K., DITSCHUNEIT, H.H., OPFERKUCH, R. and SEELING, W. The synthesis of GTF-like chromium coordination compounds and their in vitro action on the fat cell. In: Chromium in Nutrition and Metabolism. Shapcott, D. and Hubert, J. (eds). Elsevier/North-Holland, Amsterdam, 1979, pp. 189-197.

29. IRVING, J.T. Calcium and Phosphorus Metabolism. Academic Press, New York, 1973, pp. 25-26.

30. COOK, D.A. Availability of magnesium: Balance Studies in rats with various inorganic magnesium salts. J. Nutr. 103:1365-1370, 1973.

31. SEELIG, M.S. Magnesium Deficiency in the Pathogenesis of Disease. Plenum Medical Book Company, New York, 1980, p. 263.

32. VITERI, F.E., GARCIA-IBANEZ, R. and TORUN, B. Sodium iron NaFeEDTA as an iron fortification compound in Central America. Absorption Studies. Am. J. Clin. Nutr. 31:961-971, 1978.

33. MONSEN, E.R. and PAGE, J.F. Effects of EDTA and ascorbic acid on the absorption of iron from an isolated rat intestinal loop. J. Agric. Food Chem. 26:223-226, 1978.

34. CHEN, N.S.C., TSAI, A. and DYER, A.I. Effect of chelating agents on Cr absorption in rats. J. Nutr. 103: 1182-1186, 1973.